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Lead induced alterations of immunologic reactivity in mice

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INTRODUCTION

Preamble

The significance of heavy metals to the health of man and animals has been a subject for extensive speculation. Review of the literature clearly points out that excessive intake of lead and its compounds may lead to clinical toxicosis and death. Cadmium and mercury have also been implicated as heavy metals hazardous to the public health. According to Chisolm (1971), "among the natural substances that man concentrates in his immediate environment, lead is one of the most ubiquitous". The scientific literature is replete with reports on the toxic manifestations of lead poisoning; however, the significance of quantities of lead that do not produce clinical toxicity has remained a subject for extensive speculation by public health officials. Included in this speculation have been suggestions that subclinical levels of lead and other heavy metals may produce adverse effects on man and animals. Unfortunately, the specific effects of lead at subclinical levels upon the mammalian system have not been fully determined. Yet, it is clearly recognized that a large percentage of our human and animal populations is constantly exposed to appreciable quantities of this heavy metal.

Vertebrates normally are equipped with powers of resistance which are of fundamental importance to survival of the individual. This resistance or immunity, as it is called,

resides in part in the cellular elements of tissues and in part in antibodies in the blood stream. The immune system is not only well organized but is highly efficient and is thought to be mediated by a complex interaction of natural and acquired immunologic factors. Lead may alter or provoke an impairment in one or more of the major mechanisms of immunity, thereby producing immunologic deficiency disorders in susceptible human and animal populations. Such induced deficiency disorders could result in diminishing resistance to infection with organisms which previously posed little danger. The health of man and animals may, indeed, be jeopardized to a far greater extent than now realized due to heavy metals reducing their resistance to various infectious agents. Little attention, thus far, has been directed toward determining the effects and influence of lead on the resistance to bacterial invasion and immunologic reactivity of susceptible hosts.

Williams et al. (1954) suggested that lead may inactivate antibodies and thereby interfere with mechanisms whereby man and animals resist infectious disease. Their conclusions were based on the study of an acute, fatal illness in a twenty-three-month-old child with a history of eating paint. Neuropathologic findings were so similar to those seen in acute septicemia that, in spite of concentrations of lead of 0.348

mg per 100 ml of blood, it was assumed that death of the child was due to bacterial agents that were able to grow uninhibited as a result of lead-mediated antibody inactivation. Other investigators have suggested that lead exposure might interfere with one or more specific immunologic mechanisms (Hemphill et al. 1971, Palmieniak and Smolik, 1963; Rakhimova, 1968; Soliman et al., 1970; Sroczynski et al., 1964; Tepper and Pfitzer, 1970). Binding of serum proteins and gamma-globulins, for example, could remove antibodies from the circulation. Fonzi et al. (1967) reported that lead-treated animals, subsequently subjected to procedures of active immunization, developed lesser quantities of gamma-globulin; complement levels as well as anti-typhoid antibody titers were lower. Recent evidence indicates that lead may bind to various enzymes (Tepper and Pfitzer, 1970) and thus could possibly interfere with physiologic processes. Further, lead could interfere with antibody synthesis by binding with nucleic acids which may be significant in the inductive phase of antibody formation. Evidence for a specific effect of lead upon the mammalian system, when absorbed in quantities that do not produce clinical disease, has not yet been reported. It is reasonable to assume, however, that at some point short of clinical lead poisoning, in the usual sense, body concentrations of lead will reach a magnitude sufficient to cause significant changes in normal physiologic mechanisms (Tepper and

Pfitzer, 1970).

The immunologic system of mammalian species is rather complex but is now well defined. Subclinical levels of lead have been demonstrated to influence this system and the model offers an excellent approach to definitive studies of the action of lead on a physiologic mechanism.

Statement of Problem

The mechanistic basis for the action of lead in increasing susceptibility to infectious agents has not been elucidated. While the pathologic and biochemical aspects of increased lead exposure have received extensive study, very limited consideration has been given to the effect of lead upon immunologic factors. Reported experimentation is limited to the work of Fonzi et al. (1967) on lowered complement levels and serum antibody titers in lead-treated animals, a few reports in the Russian literature on levels of circulating polymorphonuclear leukocytes, phagocytic function, and serum bactericidal activity in men exposed to toxic levels of lead (Rakhimova, 1968) and a single report on the in vitro binding of antibodies by lead (Williams et al., 1954). Elucidation of the mechanism(s) whereby lead reduces resistance to bacterial infection would provide evidence of a specific activity of lead on physiologic processes. A more comprehensive approach to treatment of cases of lead exposure could result. In addition to the therapeutic approach, preventive medicine might not only include measures

to reduce exposure to lead but also prophylactic procedures to increase resistance to infectious agents.

With these ideas in mind, the experimentation reported in this dissertation was designed to provide evidence concerning the immunologic factors affected by exposure to subclinical levels of lead. These experiments represent a detailed investigation of many preliminary observations. They have led to an evaluation of several immunologic parameters in mice to provide a more thorough comprehension of the effect of lead on the functional activity of various immunologic factors and mechanisms.

LITERATURE REVIEW

Historical Concepts of Lead Exposure

Probably more has been expounded about the effects of lead and its compounds on the human and animal system, both actual and by inference, than about any other element. Plumbism (from the Latin word for lead) or saturnism (from the alchemical term) was familiar to Greek, Roman and Arabian physicians before the Christian era. Hippocrates was credited as the first to associate exposure of man to lead with the subsequent development of clinical signs (Waldron, 1966). In the second century B.C., Nicander recognized lead poisoning and recorded its symptoms in workers that were exposed to the metal (Aub et al., 1926; Gilfillan, 1965; Morgan et al., 1966). Chronic lead poisoning was incriminated as a partial cause for the decline of ancient Rome, where the metal was deliberately put into wines and certain foods and used in interior paints, to line cooking and drinking vessels, and for many other purposes not then known to be dangerous (Gilfillan, 1965). Throughout the ages, repeated reference was made to lead and its effects, as recorded in the writings of Sir George Baker in 1767 (The Devonshire Colic), Tanquerel des Planches in 1839 (Treatise on Lead Poisoning), and Rumpel in 1834 (On Lead and Its Action on the Human Body) (McCord, 1953; Hunter, 1957). In 1922, Else Blänsdorf collected and

published three thousand references, all dealing with the various aspects of lead poisoning. A much larger bibliography was compiled in 1943 by Florence A. Brous, and hundreds of papers have appeared since. In surveys such as these, and in recent texts and monographs, clinical literature dating back to at least 1840 is constantly cited. On the other hand, the pathologic physiology and chemistry of lead, the exact mode of its absorption, the changes it undergoes in the body, the manner of its action, its storage and excretion, and the changes it produces (other than gross alterations) in the body tissues have only received intensive study in the past thirty-five years.

While much of the best modern research in plumbism has been conducted in Germany, England, the United States and the Soviet Union, the foundations of our modern knowledge of the subject were laid in France. Bonté (1764) during the eighteenth century gave excellent descriptions of the symptoms of plumbism, with special reference to the paralyses. In 1803, Mérat de Vaumartoise published a treatise on the same theme, which was considered the best of its kind as regards clinical description of the acute states of lead toxicosis. A large number of French authors contributed papers in which specific aspects were discussed.

Augustin Grisolle published an essay on the colic, describing about fifty cases as seen in white-lead workers

(Grisolle, 1835). Still more important were Grisolle's studies of lead encephalopathy (Grisolle, 1837). In describing with care the delirious, epileptiform and comatose manifestations, he left relatively little to be improved upon by his successors.

The valuable work of the early French students of plumbism culminated in the epoch-making treatise of L. Tanquerel des Planches (1839). Tanquerel carried out his work in the Charité, long famous for its treatment of metallic colic. His comprehensive statistical study was made possible by the abundance of clinical material in the Charité wards, where 1217 cases of lead colic were treated between 1831 and 1839. White-lead work was responsible for 406 of the cases, painting for 382, red-lead for 75, color grinding for 68, potteries for 61, lapidaries for 35, refining for 25, German glazed card making for 11, making lead salts for 10, and fifteen (15) other trades had less than 10 each. Arthralgia was observed in 755 cases, paralysis in 127, and encephalopathy in 72 (Tanquerel des Planches, 1839). Not only did Tanquerel report in this way on the occupational aspects of lead poisoning, he also supplied comparative numerical studies of the occurrence of different symptoms. Tanquerel's clinical observations in man were very accurate and not much of importance has since been added to them. This is best appreciated by noting how frequently his century-old treatise

is still quoted in the literature of today.

It was not until the nineteenth century that the various "endemic" colics with the characteristics of lead colic began to be generally appreciated for what they really were. For the long delay three chief reasons are suggested: (1) failure to appreciate the importance of idiosyncracy, a still unexplained phenomenon, in the incidence of lead poisoning; (2) the difficulty of tracing the toxic metal when absorbed in small amounts, often from unsuspected sources; (3) the absence of easily recognizable signs in the pathologic anatomy of plumbism. Practically all of the early reports on the pathologic anatomy of plumbism were extremely variable and they undoubtedly included many abdominal inflammatory conditions and cerebral pathology of a nonspecific kind or of a different etiology. There were also, however, reports of completely negative findings. Gabriel Andral (1839) reported post-mortem observations on ten patients who had died with plumbism (although in several instances death was due to trauma or other causes). He found no significant changes and, in at least two cases where death was apparently the consequence of acute lead poisoning, he discerned "aucune lesion appreciable sur le cadavre". This was confirmed on a very large number of cases by Tanquerel des Planches (1839). The lack of correlation between the clinical picture of lead poisoning and the pathologic findings was such that a post-

mortem examination was of little or no value in proving lead to be the immediate cause of death (Alcock, 1905).

Industrial lead poisoning has been largely controlled, but accidental poisoning of children is still prevalent (Perlstein and Attala, 1966; Chisolm, 1971). Renewed interest in lead poisoning in the past ten years has led to more frequent diagnoses and recognition of the disease as a serious public health menace. Lead poisoning is reportedly the most common type of fatal poisoning in man (Perlstein and Attala, 1966). Most poisoning results from eating lead-containing paint or plaster and occurs more frequently in city slums than elsewhere. Of the children who recover from lead poisoning, it is estimated that up to 50% have persistent cerebral damage ranging from subtle learning deficits to profound mental incompetence and epilepsy (Chisolm, 1971).

Lead is found widely in animal and vegetable tissues, water, atmosphere, and is a normal constituent of the human diet. Intake and absorption of lead by the general population is therefore inevitable. Excessive absorption may result in systemic poisoning. The present degree of exposure to lead of citizens of the United States has been termed "chronic lead insult" and Patterson (1965) has suggested that the present rate of lead intake may already be sufficiently great to impair health. He maintains that the concentration of lead in the blood of "normal" American citizens is ap-

proaching that at which symptoms of chronic poisoning may be expected and that the "intellectual irritability" associated with lead poisoning might occur to a lesser but still significant extent in persons who are subject to chronic lead insult.

Physiopathology of Lead

Until recent years, when more sophisticated tests for aberrations in the metabolism of hemoglobin derivatives (hemosiderin, bilirubin and porphyrin) became available, the chief criteria of lead poisoning have been (1) clinical signs and symptoms, (2) the blood changes mentioned above, and (3) tissue levels of lead.

Since lead in the feces may never have been absorbed, chief reliance has been placed on the detection of lead in the urine. Apparently the earliest reports of the diagnosis of lead poisoning in obscure cases by this means were two papers published in 1883 by J. J. Putnam of Boston (Putnam, 1883). Patients who showed no lead line, no colic, no emaciation, and no typical extensor paralysis were observed to excrete urinary lead. The presence of lead in the urine was regarded by most authorities as indicative of lead poisoning until 1926, when Kehoe and his associates introduced the concept of "normal lead excretion" (Kehoe et al., 1926). In view of the fact that exposure to lead compounds has significant meaning only if lead absorption occurs, numerous studies

have since been done on urine and blood to provide quantitative information on the relationship between exposure and absorption.

While a great many other pathologic changes have been described, none can be selected as pathognomonic. A combination of those more frequently observed is sometimes advanced in support of a diagnosis, but the chief importance of such changes has been to suggest starting points for research in pathologic physiology.

In a list of nearly forty types of lesions said to occur in those suffering from plumbism, Aub and his colleagues (1926) followed by Cantarow and Trumper (1944) selected only two as "lesions found constantly, due to lead." These lesions are the "blue line" of the gums and anemia, together with stippling of the erythrocytes. The "blue line" or "lead line" is considered to be a reliable indicator when found, but the lead line usually fails to develop if the teeth, gums and mouth are cleaned regularly. In 1910, Dr. Alice Hamilton regarded the line as an almost invariable occurrence; between 1930 and 1940 she came to consider it relatively rare (Hamilton, 1943). This observation was regarded as an indication of changing habits in oral hygiene, since the phenomenon was still observed where poor oral hygiene and plumbism coincided (Hamilton, 1943).

The presence of basophilic granules in certain red blood

cells was first pointed out by Ehrlich (1885). He did not, however, remark on its association with lead poisoning. Credit for this correlation is usually granted solely to Behrend (1899). The value of punctate stippling as a diagnostic sign of plumbism has been much debated, and the proportion of cases in which it occurs has been variously reported. While its absence is not considered as evidence of freedom from lead absorption, its presence is generally accepted as a diagnostic indication of prime importance both in animals and in man (Zook et al., 1969).

A less specific indicator, anemia, has also been reported in the voluminous literature of lead poisoning (Aub et al., 1926; Eriksen, 1955; Goldberg et al., 1956; Hutchison and Stark, 1961). The diminution in the number of erythrocytes has been confirmed in well-developed cases of lead intoxication, but is less striking than the decrease in hemoglobin. The "blood picture" has been clarified, but the pathogenesis of these changes is not yet entirely clear.

Numerous studies in more recent times have well characterized lead poisoning in man and animals and have attempted to explain much of the pathogenesis of the disease. Lead poisoning has been reported in dogs, cattle, horses, waterfowl, sheep, swine, mink, foxes, and cats. All species of animals are susceptible; but due to eating habits or greater sensitivity, lead poisoning is more frequently observed in

cattle, horses, waterfowl, and dogs. Young animals are more susceptible than mature animals. Goats, chickens, and pigs are more tolerant (Buck et al., 1973; Radeleff, 1964; Link and Pensinger, 1966).

Lead poisoning has received extensive study in the dog, probably due to a high incidence of the disease in this species. Many cases of lead poisoning in dogs have been reported from Africa (Scott, 1963) and from New Zealand (Dodd and Staples, 1956), where the disease is "very common". It is also considered quite common in Britain (Wilson and Lewis, 1963). Lead poisoning in dogs is also widespread throughout the United States (Zook et al., 1969; Bond and Kubin, 1949; Estrada, 1962; Lieberman, 1948; Molpus, 1958; Pettit et al., 1956). Zook et al., (1969) reported on 42 cases of accidental lead poisoning in dogs where age was given, 31 were less than one year old, and the rest were from 15 months to 11 years of age. It is widely believed that young dogs are more susceptible to lead poisoning than are old dogs, but this opinion seems to be based on rather tenuous evidence. It appears that dogs are rather commonly poisoned with lead, but this may be related to their bizarre eating habits resulting in the ingestion of lead-containing substances (Zook et al., 1969).

Clinical signs of lead poisoning in dogs are usually reported as beginning with anorexia, vomiting, colic, diarrhea

or constipation, and often progressing to signs of nervous damage such as hyperexcitability, hysterical barking, champing fits, convulsions, opisthotonus, paraplegia, muscular spasm, hyperesthesia, blindness, or behavioral changes (Bond and Kubin, 1949; Calvery et al., 1938; Dodd and Staples, 1956; Estrada, 1962; Fauts and Page, 1942; Lieberman, 1948; Mitchell, 1940; Molpus, 1958; Monot, 1961; Oliver et al., 1959; Pettit et al., 1956; Scott, 1963; Staples, 1955; Wilson and Lewis, 1963; Zook et al., 1969). Other signs mentioned include weakness, weight loss, sialosis, and scurfy skin (Calvery et al., 1938; Lieberman, 1948; Wilson and Lewis, 1963). Of a total of about 76 accidental and 51 experimental lead poisonings of dogs, a lead-line or Burtonian line was reported on the gums in only 3 dogs (Zook et al., 1969). Discharges from the eyes and nose are occasionally reported, but these signs may be the result of concurrent infection. It has been reported that a number of dogs apparently suffered from distemper and lead poisoning simultaneously (Zook et al., 1969).

Acute lethal single exposures are usually considered to be in the magnitude of 400-600 mg per kg in calves and 600-800 mg per kg in adult cattle. However, a case has been reported in dairy cattle where an estimated single exposure of 4.8 mg per kg red oxide of lead in paint killed 18 of 20 animals. The daily intake of approximately 6-7 mg per kg body weight appears to be the minimum dose which will eventually

give rise to toxicosis in horses and cattle. Horses are somewhat more susceptible than cattle (Hammond and Aronson, 1964). Sheep were fed metallic lead daily during gestation at levels ranging from 0.5-16.0 mg per kg to maintain blood lead levels of 0.4-0.6 ppm. Severe nonfatal poisoning occurred, manifested by anorexia, emaciation, and abortion (Sharma, 1971).

Although lead is poorly absorbed from the digestive tract, blood lead levels may rise to 2.5-4.0 ppm within 12 hours of ingestion and decline to 1-1.5 ppm in 48-72 hours. However, the blood level will remain elevated for one to two months (Allcroft, 1951). This slow decline in blood lead level emphasizes the slow excretion of lead in untreated animals and the role of lead as a chronic cumulative toxicant.

Lead accumulates in the body so that chronic exposure to small amounts may lead to toxicosis. Silage containing 140 ppm lead has poisoned cattle. Herbage grown on lead-contaminated soil has contained 260-914 ppm and caused death in calves. Normal herbage should contain 3-7 ppm or less. Forage with levels less than 40-60 ppm did not cause problems in lambs (Buck et al., 1973).

Lead appears to affect all major organs. Circulating lead combines with erythrocytes and, unless in very high concentrations, is not found in plasma. Anemia may result initially from an increased fragility of red cells which leads

to premature destruction and, secondly, by depression of bone marrow so that fewer cells are produced (Aub et al., 1926). The nervous system is affected by a decreased blood supply due to damage to capillaries, which results in either edema or a collapse of the small arteries. Peripheral nerves are affected by a segmental demyelination which interferes with nerve conduction (Gombault, 1880). The roaring that is observed in horses, the pharyngeal or buccal paralysis in cattle, and the paralysis of the masseter muscles in dogs are evidence of neurological damage of either cranial nerves or brain stem nuclei (Christian and Tryphonas, 1971).

In the kidney, lead causes degeneration and necrosis of mitochondria and renal tubule cells (Watrach, 1964; Goyer, 1968).

Additional evidence which may be used to substantiate excessive lead exposure is the presence of acid-fast intranuclear inclusions in the proximal convoluted tubules of the kidneys and the presence of significant amounts of lead in tissues. Acid-fast intranuclear inclusions, however, are not always present and are not specific (Jubb and Kennedy, 1963; Christian and Tryphonas, 1971). In acute exposures, lead may cause necrosis of gastrointestinal mucosa. Liver degeneration and necrosis can follow both acute and chronic exposures (Hatch and Funnell, 1969). In young animals, lead will accumulate in metabolically active growth centers of long

bones (Clegg and Rylands, 1966). This results in increased densities on radiographs. Experimentally, lead will suppress growth in young animals. Lead crosses the placental barrier, and the fetal liver can accumulate toxic levels. Lead can be a cause of abortion, fetal resorption, and sterility (Allcroft, 1951).

Lead is removed slowly from the body, primarily by the kidneys. For instance, sheep can excrete a maximum of 0.8 mg lead per day in the urine. Four to six months may be required to reduce the blood lead levels to control levels following acute nonfatal exposure. The magnitude of biliary excretion of lead is poorly understood. Lead is also excreted in the milk which is a potential source of the metal for nursing animals (Hammond and Aronson, 1964).

The subcellular effects of lead have not been fully investigated. However, lead will cause rupture of lysosomes and release of acid phosphatase which is required for energy production and protein synthesis. Lead interferes with several enzymes involved in heme synthesis (Brun and Brunk, 1967). One important effect is inhibition of aminolevulinic acid dehydrase (ALAD), thus blocking the metabolism of aminolevulinic acid (ALA), causing abnormally large amounts of delta-aminolevulinic acid (Δ-ALA) to appear in the plasma and urine (Blumenthal et al., 1972). The detection of abnormal levels of Δ-ALA in urine has been used as a diagnostic tool in human

and veterinary medicine. Lead also blocks the incorporation of iron into the heme molecule. In general, it is thought that lead will interfere with thiol-containing enzymes (-SH) (Bessis and Breton-Gorius, 1959).

Lead has been found to enhance susceptibility of rats to bacterial endotoxin. It was suggested in this study that lead might increase endotoxin sensitivity by inactivating the reticuloendothelial system (Selye et al., 1966). Truscott (1970) studied the effect of lead acetate on endotoxin toxicity in chicks. His findings were similar to those of Selye et al., (1966), that lead potentiates the toxic effect of endotoxin.

Binding of serum proteins by lead was reported by Matsukuto (1959). Lead-210 containing solution was incubated with human serum. Radioactivity was observed in the albumin and α 2-globulin fractions of rats injected with Lead-210.

Lead has been shown to reduce the resistance of mice to bacterial infection by inhibiting antibody production or tying up antibodies already produced even at exposure levels insufficient to cause other signs of poisoning (Hemphill et al., 1971). This finding could have profound influence upon the present attitude toward environmental contamination by lead, if similar effects of lead are shown in other species of animals.

Distribution and Availability of Lead

Production and utilization

Lead is produced by melting the ore galena (lead sulphide). This substance is widely distributed and occurs in both surface and underground deposits. Production figures are not the best guide to utilization since there is considerable recycling of this valuable metal as scrap through secondary smelters (Barltrop, 1969). The production and consumption figures for the United States have been reported in detail and concern some 1,100,000 short tons per annum, of which about 60% is metallic lead and the remainder is in the wide variety of lead compounds (Ziegfeld, 1964). The major industries utilizing lead are listed in Table 1, together with their percentage share of the total annual consumption.

Table 1. Lead consumption by industry in the United States (after Ziegfeld, 1964)

Industry	%
Storage batteries	35
Petrol	16
Building	11
Brass, ceramics, type set, paint	15
Other	23

From the figures cited (Ziegfeld, 1964), it is particularly interesting that 175,000 tons of lead is utilized in the production of petrol, since much of this will eventually be distributed into the atmosphere as automobile exhaust. In addition, 2,500 tons of lead arsenate are intended for application to food-crops as pesticide.

Among the many present day uses for lead are some which may not be easily recognized, e.g., lead compounds as stabilizers in certain plastics. New uses are continually sought and the application of organo-leads as biocides in wood and fabric preservation and even bilharzial control are being studied (Van der Kerk, 1966). Although organic compounds of lead have their own toxicity characteristic, ultimately they are all likely to be degraded and to release lead in the inorganic form.

The industrial contribution to environmental lead may result from the emission of lead particles into the atmosphere and by pollution of natural waters with lead-containing industrial-effluents. Polluted atmosphere and water both serve to contaminate soil, plants and foodstuffs. Thus, both atmosphere and diet contribute to the daily lead intake (Barltrop, 1969).

Atmospheric lead

Since their introduction into petrol in 1923, nearly 3 million tons of lead alkyls have been marketed (Chow and

Johnstone, 1965). After combustion, the anti-knock derivatives in petrol are released in organic form as a mixture of halides and oxides. Atmospheric pollution has occurred on a world scale and has affected even remote regions. Serial samples of earth and snow since 1900 have shown a sharp increase in lead content during recent decades (Murozumi, et al., 1966). The blood lead of persons living close to regions of high traffic density such as motorways has been shown to be significantly higher (22.7 µg/100 g) than that of comparable individuals in an area remote from heavy traffic 16.0 µg/100 g).

Although automobile exhaust fumes are a major problem in the Northern Hemisphere, industrial undertakings also make a significant contribution to atmospheric pollution. In areas close to lead smelters or installations for the burning and distillation of coal, significant atmospheric pollution with lead may occur and should be considered with regard to the local population (Barltrop, 1969). It is seldom realized that the atmospheric emissions from smelters may be very large; Chakraborty et al., (1964) reported one plant in India that discharged 2,000 pounds (907 kg) of lead into the atmosphere daily. A similar example in Minnesota deposited sufficient lead on local foliage to precipitate acute lead poisoning in the local cattle and horses that consumed it (Hammond and Aronson, 1964).

Water

Natural waters contain lead derived from a number of sources including rocks and soil, particulate matter derived from atmospheric fallout and, in some instances, a contribution from sewage. Variations in the lead content of waters occur between different geographic regions and surface water has been found to contain 4-20 µg per liter. These figures contrast with those of well water consumed by a child with 'epilepsy' which had a lead content of 408 µg per liter (Oyanguren and Perez, 1966).

Contamination of reservoirs by air pollution is probably not yet a major problem. However, in the Seattle, Washington region, it has been established that about 70 mg of soluble lead derived largely from road traffic exhaust rather than industrial sources, settles on each square meter of surface per month. The large volume of the reservoirs minimizes the effect of this fallout. In the winter, however, decreased utilization of water may result in a doubling of the lead content to 6 µg per liter (Johnson, et al., 1966). Recreational motor boats disperse 4×10^8 g of lead into natural waters in the United States each year from combustion of fuel containing lead alkyls (English, cited in Ettinger, 1967), but this is a small contribution compared with sewage which contributes a further 8×10^9 g per annum.

The use of lead pipe, lead solder on copper pipe and occasionally even lead paint on water storage tanks, gives rise in many countries to a situation in which the lead content of the water supplies may differ greatly from the water actually consumed. Factors which influence the "plumbosolvency" of a given water include the pH and the calcium content. Soft acid waters are especially liable to dissolve lead with which they come into contact. Prolonged standing in a pipe may result in some lead being dissolved and water in the terminal lead pipes of a dwelling will have an appreciably higher lead content after standing overnight. The World Health Organization (WHO) revised 1963 standard for drinking water set an upper limit of 50 µg per liter for lead, but the WHO "European" standard allows drinking water to contain up to 300 µg per liter after standing in the pipe for 16 hours (Barltrop, 1969).

Radioactive lead

Radioactive lead is continuously deposited on the earth's surface as part of the "natural" fallout, although it is increased after atmospheric nuclear detonations. Rocks containing radium continually liberate radon into the atmosphere, which in turn decays through a series of shortlived isotopes to Lead-210. This isotope has a half-life of 22 years and ultimately decays through a further series of isotopes to

stable lead. It has been calculated that 22 x 10^{24} atoms of Lead-210 are distributed over the earth's surface in this way each year (Lambert and Nezami, 1961). Of significance here is the fact that the body cannot distinguish radioactive from stable lead and, since this is a bone-seeking element, the situation is analogous to that of the more familiar Strontium-90. It has been shown that there is a close correlation between the Strontium-90 and the Lead-210 content of human bones (Barltrop, 1969).

Foodstuffs

The contribution of foodstuffs to the daily lead burden of the adult is of the order of 300 µg per day, but this is prone to local and geographical variation due to differences in preparation and distribution (Kehoe, et al., 1933). Some animal tissues, such as liver and kidney, have a special affinity for lead so that the intake of lead from a diet rich in these might be higher than an equivalent diet based upon other tissues derived from the same animal (Kehoe et al., 1933).

Plants and vegetables that are grown near dense traffic routes are liable to contamination. Analyses of leaves taken from sites near highways in Great Britain have revealed mean lead contents of 87 mg per kg which is almost double the mean lead content of foliage from sites remote from traffic (45 mg/

kg). The results were unrelated to the lead content of the soil in which the plants grew (Everett, Day and Reynolds, 1967). A seasonal variation in the lead content of pasture herbage has been described by Mitchell and Reith (1966). During senescence a redistribution of lead ions occurs within the plant so that the above-ground portion may reach concentrations of 30-40 mg per kg during the winter compared with 0.3-1.5 mg per kg in the spring. Levels of 40 mg per kg are considered of importance to animals feeding on this material (Mitchell and Reith, 1966).

The use of lead-containing pesticides has already been cited, especially the use of lead arsenate sprays. Associated with the use of lead arsenate sprays, elevated lead concentrations have been found in fruit, tomatoes and coffee beans (Barltrop, 1969), and apples (Pocklington and Tatton, 1966). Studies on imported apples by the latter authors indicated that increased lead residues were present in apples from countries that allow the use of lead arsenate sprays compared with countries that do not. Thus, apples imported from Canada where lead arsenate sprays are used contained as much as 1750 µg per kg, whereas South African apples contained only 100-300 µg per kg.

The continued exposure of sections of the population to increased environmental lead levels remains a subject for extensive speculation and in the words of Chisolm (1971), "al-

though no population group is apparently yet being subjected to levels of exposure associated with the symptoms of lead poisoning, it is clear that a continued rise in the pollution of the environment with lead could eventually produce levels of exposure that could have adverse affects on human and animal health."

Today our concerns about human and animal health and the dissemination of lead into the environment are twofold: (1) there is a need to know whether or not the current level of lead absorption in the general population presents some subtle risk to health; (2) there is even more urgent need to control this hazard in the several subgroups within the general population that run the risk of clinical plumbism and its known consequences (Chisolm, 1971). Increased exposure to lead has been shown in populations exposed to lead as an environmental pollutant.

The magnitude of present day environmental lead contamination is emphasized by the recent calculation that 20 tons of lead are consumed annually by the population of the United States (Patterson, 1965). Some natural environmental lead is radioactive Lead-210 and this provides an additional hazard for the total body lead burden.

Concepts of Immunity

Immunity may be defined as the state of being able to resist and/or overcome harmful agents or influences (Weiser et al., 1969). The science of immunology encompasses a study of immunity and is a direct outgrowth of the study of host responses to infectious agents. In fact, the early successes of bacteriology are the foundations upon which modern immunology rests. At the present time, however, immunology has evolved as a true science and encompasses the study of immunologic mechanisms in relation to disease processes, in the causation of disease, and as an experimental method in association with other sciences. This treatise primarily relates to a role for the immune processes in resisting bacterial infection. Therefore, the following discussion will be restricted to mechanisms associated with resistance to infectious disease.

Since late in the nineteenth century, there has been an understanding that both cellular and humoral factors are associated with resistance. Knowledge of the basic cellular mechanisms dates from the classical discovery of phagocytosis by Metchnikoff (Hirsch, 1965) and the description of the delayed-hypersensitivity reaction by Koch (Weiser et al., 1969). Knowledge of the involvement of humoral factors dates from the work of Buchner, von Behring, Erhlich and others

around 1890 (Weiser et al., 1969). Primary emphasis during the early twentieth century was on humoral factors. This restriction of interest was due to a primary concern with the protective role of serum antibody, particularly as related to antitoxic immunity. Also, the discovery of complement by Bordet in 1899 (Bordet, 1899) strengthened the concept of humoral protective factors in immunity.

It is now known, however, that cellular mechanisms are also involved in the immune response to infectious agents and these play perhaps the most critical and decisive role in certain host-parasite interactions. Thus, immunity must include a very broad biologic view encompassing the vast interplay of humoral and cellular factors which comprise the total immunologic responses of the host (Bellanti, 1971). The cellular-humoral dichotomy is illustrated by the clinical observations of increased susceptibility to infection seen in individuals with congenital defects of the immunologic system (Good, 1971). Some individuals lack the humoral protective function but retain the cellular; others are deficient in cellular but have normal humoral activity; still others are defective in both humoral and cellular functions. Clearly both areas are of profound importance and both cellular and humoral factors are interrelated and interdependent.

With due recognition of the interrelationships, immunity will be categorized for purposes of discussion as (1) natural,

(2) acquired, (3) cell-mediated, and (4) humoral.

Natural immunity

The resistance which an individual possesses even before contact with a pathogenic organism is called innate or natural immunity and can involve cellular as well as humoral aspects. This form of immunity has a genetic basis and is subject to species and individual variation (Gowen, 1960). Many aspects of the functional basis for innate immunity are unknown; moreover, it is extremely difficult to distinguish between defense factors that represent innate and acquired immunity. As a consequence, although some of the mechanisms are recognized, innate immunity is not well-defined and is difficult to accurately quantitate at the present time (Raffel, 1961).

Acquired immunity

Acquired immunity is the resistance which develops as a result of contact with the organism in question or is the result of transfer of immune substances from another individual. Acquired immunity tends to possess a degree of specificity not associated with natural immunity but may be either cellular or humoral in character. Specific immunity that is acquired actively by natural means usually develops as a result of either an apparent or inapparent encounter with a parasite or with related organisms. On the other hand, immunity that is acquired passively by natural means involves the transfer of

a specific immune globulin (antibody) from the mother to the fetus or neonate by way of the placenta or colostrum (Brambell, 1970). Apparently, acquired passive immunity in man is, for the most part, largely mediated by specific antibody of the class immunoglobulin-G because such immunity is transferred almost exclusively to the fetus by way of the placenta. However, in other species of animals, including laboratory and domestic animals, antibody of various classes may be transferred via colostrum (Brambell, 1970).

Immunity that is acquired actively by artificial means is the result of purposeful vaccination with immunogenic antigen(s) derived from or associated with a specific microorganism. Immunity that is acquired passively by artificial means is accomplished by the injection of an immune serum which contains globulins capable of conveying specific immunity against a given microorganism.

Cell-mediated immunity

The term "cell-mediated immunity" will be used to describe those resistance mechanisms in which cells play a major role. This terminology is preferable to "cellular immunity" which according to Mackaness and Blanden "will be found written with quotation marks, as if to signify ambiguity or doubt in the mind of the author. There is reason for this, for the concept is vague, the underlying immunological

processes are ill-defined, and the term has been used with two different connotations" (Mackaness and Blanden, 1970). Cell-mediated immunity relates to the immunologic processes involving phagocytic cells and specifically sensitized lymphocytes.

The phagocytic cells of the mammalian host comprise one of the very important defense mechanisms against invading microorganisms. Phagocytic cells are grouped into two basic classes: (a) polymorphonuclear phagocytes found in marrow, blood, and tissues, and (b) mononuclear phagocytes, including blood monocytes and macrophages of tissues and various organs such as the liver, spleen, lymph glands, and bone marrow (Hirsch, 1965). When invading microorganisms pass the natural barriers such as skin and mucous membranes, they may be acted upon by the defense mechanisms of phagocytosis and inflammation. In the tissues, polymorphonuclear neutrophils (PMNs) and macrophages attracted to the site by an inflammatory response may engulf and destroy the invading microorganisms. Foreign materials reaching the blood or lymphatic system are subsequently engulfed by PMNs or filtered out by phagocytic cells of the reticuloendothelial system (RES). The RES involves cells of the spleen, thymus, liver and other lymphoid tissues and acts to remove effete and worn-out cells, to remove foreign materials, and probably plays a role in antibody formation, particularly in lymph nodes (Carpenter, 1965).

Phagocytosis is enhanced by other factors associated with the immunologic system such as antibody, complement and immunoconglutinin. Macrophages are able to interact with antibodies which have an affinity for certain receptors on the membranes of these cells. This antibody is denoted as cytophilic for macrophages, because of its exceptionally strong affinity for their membranes (Berken and Benacerraf, 1966; Gordon and Ford, 1971). Macrophages containing cytophilic antibody have definitely enhanced abilities to recognize, engulf and destroy antigenic micro-organisms, or to restrict their intracellular growth (Gordon and Ford, 1971).

Macrophages are also an important contributor to acquired resistance. With certain types of chronic bacterial infections, immune phagocytes develop which have an increased ability to engulf and destroy bacterial organisms (Fong et al., 1964; Elberg, 1960). This type of resistance is established only following contact with living bacterial organisms or as a result of experimental transfer of nucleic acid rich fractions of immune macrophages (Fong et al., 1964).

Exemplifying one of the most notable features of acquired cellular resistance is the inactivation of S. typhimurium in the phagocytic cells of actively infected mice which commences almost immediately after ingestion and proceeds rapidly until virtually every intracellular organism has been killed. This microbicidal effect is not due to specific anti-bacterial

antibody because cells from animals infected with Listeria monocytogenes or S. typhimurium are equally microbicidal for S. typhimurium despite the absence of demonstrable anti-Salmonella antibody either in the serum or adsorbed to the cells of the Listeria-infected mice (Mackaness, 1970; Mackaness, 1971b).

Recently, the term "cellular immunity" has been applied to resistance phenomena associated with sensitized lymphocytes (Mackaness, 1970). The sensitized lymphocyte serves as a mediator of a number of reactions. These small-to-medium lymphocytes that have become sensitized to the antigen by previous exposure act to recognize the antigen. The molecular basis of antigen recognition by sensitized lymphocytes is not clearly understood but must be mediated by receptors on their surface (Bach et al., 1971). Classical immunoglobulins have not been demonstrated to have a role in this recognition, but nonetheless, cellular recognition of antigen is highly specific (Bach et al., 1971; Gordon and Ford, 1971). This type of immunity involves specifically sensitized lymphocytes and the mechanism appears to be monitored by the thymus and mediated by the thymic dependent group of lymphoreticular tissues (Bellanti, 1971). The sensitized lymphocyte serves as a mediating cell in immunologic responses such as enhanced phagocytosis, delayed hypersensitivity and the rejection of foreign grafts and malignant cells (Mackaness, 1971a). Reaction

of a sensitized lymphocyte with its target antigen results in the activation of the sensitized cell, stimulating it to release substances which affect macrophages (Mackaness, 1971b). One of these substances is chemotactic for circulating blood monocytes, inducing these cells to emigrate through the walls of vessels and accumulate in the region of the activated lymphocyte, assuming the form of tissue macrophages or histiocytes. Another substance (macrophage inhibiting factor) released from activated lymphocytes inhibits the migration of macrophages in proximity to the lymphocyte, preventing these cells from leaving the site. Histological section of such sites, therefore, shows massive infiltration of mononuclear phagocytes. These macrophages are responsible for many of the features of cellular reactions (Mackaness, 1971b; Gordon and Ford, 1971; Nelson, 1969).

Humoral immunity

Resistance to disease processes or conditions can be mediated by a number of humoral factors either normally present in the animal body or produced by contact with or exposure to antigen(s). These factors may act alone or commonly act in concert to establish or produce resistance to infectious agents. Among the humoral resistance factors are included antibodies, complement, properdin, beta-lysin, lysozyme and immuno-conglutinin.

Antibodies An antibody is "a substance (commonly, if not always, a gamma globulin) which can be incited in an animal by an antigen or by hapten combined with a carrier and which reacts specifically with the antigen or hapten. Some antibodies occur naturally without known antigen stimulation" (Weiser et al., 1969). Antibodies are a heterogeneous mixture of proteins found in the gamma and beta globulin fractions of serum. Antibodies occur as a variety of classes and subclasses of globulin molecules (immunoglobulins). Antibodies have been described in terms of some functional parameter of interaction with antigen as precipitins, lysins, antitoxins, agglutinins and so on, and it was previously thought that these effects were due to the same kind of antibody acting in different circumstances (Humphrey, 1967). It is now clear that not all antibodies reacting with the same antigen are of the same kind; they may differ in function as well as in their biologic properties (Holborow, 1968; Raffel, 1961).

Natural antibody Substances that react with an antigen may occur in the blood of animals which have not been immunized and, presumably, have had no contact with the antigen. Among these antigens are erythrocytes of different individuals of the same species. These substances, very similar to induced antibodies in their behavior, are called natural or normal antibodies. Although some so-called normal antibodies can, in

fact, be accounted for by antigenic stimulation, the presence of many natural antibodies cannot be satisfactorily explained at the present time.

A prime example of a class of normal antibodies is the isoagglutinins of the human blood groups (Race and Sanger, 1962). Although the presence or absence of these antibodies is genetically dependent, their mode of origin is still controversial (Wiener, 1951; Abramoff and La Via, 1970). Antibodies to certain bacteria have been demonstrated without apparent antigenic stimulation (Boyden, 1966; Skarnes and Watson, 1957; Shilo, 1959). Jerne (1956) described natural antibodies against bacteriophages; however, the most extensively studied natural antibodies have been those reactive with pathogenic bacteria and those responsible for blood group incompatibility (Wilson and Miles, 1955; Skarnes and Watson, 1957). When present in serum, natural antibodies are low in titer, but they may exercise a significant role in conferring resistance to certain infections.

Acquired antibody Acquired antibodies are specific globulins produced by the body in response to contact with an antigen (Raffel, 1961; Carpenter, 1965). Encounter with an antigen leads to a series of metabolic and cellular phenomena culminating in the elaboration of antibody molecules capable of reacting with the antigen.

The mechanism of antibody formation has been a subject for extensive study in recent years, but all aspects have not been clearly elucidated. It is apparent that synthesis of specific antibody takes place in lymphocytes and plasma cells (Henriksen, 1970). These specialized cells of the lymphoreticular tissues synthesize gamma globulins in their ribosomes located on the endoplasmic reticulum of the cytoplasm (Carpenter, 1965). Therefore, actual synthesis of antibody does not differ from the normal protein synthesizing process in mammalian cells. However, the inductive phase, or the mechanism whereby cells are stimulated to elaborate a highly specific protein molecule, is not clearly understood. Antigen in some manner induces cells of the lymphoid system to differentiate and multiply so that variable quantities of antibody may be produced.

The manner of presentation of antigen appears to be critical in antibody formation; some antigens apparently require processing by macrophages and a subsequent transfer of information to antibody producing cells (Henriksen, 1970). Other antigens act directly on receptor sites located on the surface of immunocompetent lymphocytes to trigger the process of antibody formation (Mackaness, 1971a). Antibody formation is, therefore, an extremely complex process involving not only cell cooperation phenomena, but also the biochemical phenomena associated with antigen metabolism and protein synthesis.

Complement The term complement refers to a collective group of eleven blood serum proteins normally present in the blood of vertebrate species. The proteins are regarded operationally as a biological system that engages in a characteristic series of reactions in the presence of antigen-antibody complexes (Müller-Eberhard, 1969a). Complement is commonly defined on the basis of its membrane-damaging or cytolytic activity since the occurrence of the reaction termed "fixation" in the immediate vicinity of biological membranes may lead to destruction of the membrane. However, complement is now known to participate in a variety of immunologic and pathologic reactions (Müller-Eberhard, 1969b).

The presence of complement in fresh normal serum and its vibriolytic activity was initially observed by Bordet (Bordet, 1899). Complement has subsequently been shown to be a normal constituent of serum and to be globulin in character but unrelated to immunoglobulins. The concentration of complement in serum is rather constant for each species of animal, although its level may increase or decrease in certain disease states. In man, complement is present at birth, although in lower concentration than in the adult (Lepow, 1965). Its concentration is not influenced by immunization.

Since its discovery, the cytolytic activity of serum has been dissected into a growing number of components until its full complexity emerged (Müller-Eberhard, 1968). It is now

known that eleven distinct serum factors act in concert to generate the cytolytic activity of the complement system. According to a consensus of the active workers in the complement field, the components of complement (C') are designated by numerals, i.e., they are referred to as C'1, C'2, C'3, C'4, C'5, C'6, C'7, C'8, and C'9; and the three subcomponents of C'1 are called C'1q, C'1r, and C'1s. Each component is defined by its position in the complement reaction sequence and also by physical and chemical characteristics. Most importantly, it is now possible to define complement components in terms of their unique immunochemical properties, since specific antisera have been prepared to at least six of them.

In addition to causing membrane damage, complement has other biologic effects which may play a role in host defense to infections and in mechanisms of inflammation. Thus, complement and its components are involved in the potentiation of phagocytosis, opsonification, immune adherence, increased vascular permeability due to anaphylatoxin and histamine release, and white cell chemotaxis (Gerwurz, 1971; Müller-Eberhard, 1968; Jensen, 1967).

Properdin Studies by Pillemer and co-workers, (1953a) on the mechanism of inactivation of the third component of human complement by zymosan led directly to discovery of a previously unrecognized serum protein, properdin, and to the

concept of the properdin system. It had been known since the early work of von Dungern (1900) that whole yeast cells inactivate C'3. Later investigations by Whitehead, Gordon and Wormall (1925) showed that a fraction of yeast, called zymin, could be substituted for whole yeast cells. Subsequently, Pillemer and Ecker (1941) isolated a yeast product, zymosan, which was derived from the cell wall of yeast, was mainly carbohydrate in nature, and was highly active in inactivating C'3 at 37°C. It was generally assumed that C'3 was adsorbed to the surface of zymosan, a product insoluble in aqueous media, but detailed investigations had not been performed. Subsequent studies revealed that inactivation of C'3 by zymosan was not a simple adsorption but had kinetic characteristics both of a stoichiometric and an enzymatic reaction (Pillemer et al.,1953b). Furthermore, they demonstrated that Mg++, a hydrazine-sensitive serum factor resembling C'4, and a heat-labile serum factor resembling C'1 or C'2 were all required for inactivation of C'3 by zymosan. The reaction was further dependent on pH, ionic strength, temperature and time.

Detailed investigation of the effect of temperature on inactivation of C'3 by zymosan made possible the discovery of properdin (Pillemer et al., 1954). Although zymosan did not inactivate C'3 in human serum at 15°C, C'3 in the resulting supernatant serum could not be inactivated by fresh zymosan at

37°C. Adsorption of a normal serum protein, properdin, by zymosan at 15°C was demonstrated. This substance could be eluted from the zymosan complex formed at 15°C. Addition of the eluate to serum adsorbed with zymosan at 15°C and, therefore, deficient in properdin reconstituted a complete system for inactivation of C'3 by fresh zymosan at 37°C. Properdin was, therefore, defined i. terms of its requirement for inactivation of C'3 by zymosan. Serum lacking properdin (RP) was assumed to supply in excess all of the other factors required for inactivation of C'3, including Mg++ and the hydrazine-labile and heat-labile serum constituents. All of these factors considered as a group were termed the properdin system.

Properdin itself was partially purified by adsorption to and elution from zymosan under optimal conditions (Todd et al., 1959). Preparations purified 2500-fold with respect to serum were subjected to chemical, physico-chemical, biological and immunological characterization (Lepow et al., 1959). The absorption spectrum exhibited a typical protein absorption maximum at 280 mμ. At a concentration of 600 units of properdin per ml, small amounts of glucose (after acid hydrolysis) and lipid but no phosphorus were detectable. Electrophoretic analysis in pH 8.6 barbital buffer showed a major peak (90%) with the mobility of gamma 1-globulins and a second peak (10%) corresponding to gamma 2-globulins. Analysis in

the ultracentrifuge revealed four components with sedimentation constants of about 6, 9, 12 and 18S. Properdin activity was associated primarily with the fastest sedimenting component.

Ouchterlony analysis, using rabbit antiserum to partially purified human properdin, showed the presence of at least five antigens. After absorption of the rabbit antiserum with human RP, a single line was observed, probably representing properdin. A variety of trace activities was found in partially purified properdin, including anti-A and anti-B isohemagglutinins, an agglutinin for zymosan, several blood clotting factors, lysozyme and phosphatase. All of these could be shown, however, to be distinct from properdin itself (Lepow et al., 1959).

Despite the heterogeneity of partially purified properdin, the preparation displayed common activity in a variety of biological systems. Human serum deficient in properdin (RP) was not only altered with respect to inactivation of C'3 by zymosan at 37°C but was also inactive or greatly reduced in activity with respect to certain bactericidal, virus-neutralizing, hemolytic and protozoa-inactivating properties of the parent serum. These activities could be restored to RP by addition of small amounts of partially purified human properdin. These observations strengthened the original concept of the properdin system and led to the hypothesis that

the properdin system was a nonspecific humoral mechanism which might be part of the host's defenses in natural resistance. Properdin biologically functions in the bactericidal activity of normal serum against a variety of gram-negative bacteria (Wardlaw and Pillemer, 1956), the neutralizing activity of normal serum for certain viruses (Wedgwood et al., 1956), the hemolytic activity of normal serum against erythrocytes from patients with nocturnal hemoglobinuria (Hinz et al., 1956) and against erythrocytes treated with tannic acid (Hinz et al., 1957). The functional role of properdin is undoubtedly associated with the fixation of complement and recent experimentation permitted Lepow (1971) to conclude "there can no longer be reasonable doubt about the properdin system as a unique, alternative pathway to the terminal components of complement. It therefore comprises an additional mechanism for generating biologically active, complement-derived cleavage products, as well as mediating certain bactericidal, virus - neutralizing, and hemolytic properties of normal serum."

Beta-lysin Beta-lysin is an enzyme which is active against gram-positive bacterial organisms (Skarnes and Watson, 1957). Beta-lysin is usually present in low levels in normal human serum and appears in greatly increased amounts during the acute phase of infectious and other inflammatory diseases (Weiser et al., 1969). The source of the material has been

reported to be platelets and is distinct from lysozyme, complement, and antibody. It appears to be a complex system of enzymes which are very effective bactericidal agents (Donaldson et al., 1964).

Lysozyme Lysozyme is a mucolytic enzyme which has been shown to have bactericidal activity. This enzyme is found abundantly in phagocytic cells but also in body fluids (Heise and Myrvik, 1967). The enzyme functions by virtue of mucolytic properties which cleave acetyl-amino-sugars, the structural backbone of both gram-positive and gram-negative bacteria. This material appears to be an important non-specific defense mechanism of the host, may function independently and is known to accentuate the cytolytic activity of complement (Weiser, et al., 1969).

Immuno-conglutinin Immuno-conglutinin is a nonspecific humoral substance occurring in many animals, including rabbits, guinea pigs, mice, cats and man, as a result of infection with bacteria, viruses, rickettsia, and protozoa (Coombs et al., 1961). The time of increase and peak titer correspond well with the specific antibody response. In acute infections the titer slowly declines to pre-infection levels in 30 days. In chronic infections the titer may persist. In mice, production of immuno-conglutinin has been demonstrated as a result of subclinical infection (Ingram,

1959). Immuno-conglutinins are auto-antibodies against certain components of complement which often appear in the sera of human beings in whom massive antigen-antibody reactions are taking place. The functional significance of immuno-conglutinin is in the enhancement of phagocytosis (Weiser et al., 1969).

Biological amplification

The interaction of humoral and cellular factors represents a group of phenomena termed biologic amplification systems (Good et al., 1970). While a single mechanism of resistance may function to some degree in combating an infectious process, the synergistic effect of multiple factors may greatly augment the reaction. Representative of such reactions are the combined cytolytic effects of complement and lysozyme, the enhanced phagocytic activity in the presence of antibody, the further enhancement of phagocytosis in the presence of antibody, complement and immuno-conglutinins and the participation of a variety of factors in the inflammatory response.

Inflammation is a classical example of participation by multiple cellular and humoral factors. Antibody may complex with an invading microorganism; complement is fixed; fixation of complement leads to release of chemotactic factors (which attract macrophages) or anaphylatoxin (which increases vascular permeability); additional quantities of complement may lead to

bacteriolytic and bactericidal activity; attracted macrophages may engulf and destroy the microorganisms; macrophages may release lysozyme which is bacteriolytic; sensitized lymphocytes may release macrophage inhibiting factor to retain macrophages in the area; sensitized lymphocytes may transfer information to macrophages to enhance the phagocytic and microbicidal activities. This interplay between complement and other factors is the mechanistic basis for the inflammatory response.

The kallikrein system is yet another component of the amplification system and represents a well defined system of proteins in plasma, activated by a reaction of antigen with antibody. Presumably, the enzyme kallikrein interacts with its substrate to produce several vasoactive peptides; slow reactive substance and anaphylatoxin are humoral factors generated by the interaction of antigen with sensitized cells. All of these agents increase capillary permeabiltiy (Bellanti, 1971).

Factors influencing immunity

Genetic considerations The host-parasite relationship may be influenced by mechanisms which are genetically controlled. Genetic variations in the host as well as in the parasite are important factors in this relationship. In species where variations of resistance exist, individual dif-

ferences may be found which depend on genetic factors. Rich (1923) established a strain of guinea pigs deficient for complement. Later studies suggested that the deficiency was due to lack of the third component of complement (C'3) (Hyde, 1923). This strain was distinct from normal guinea pigs which possess relatively high levels of all components of complement. Genetic analysis indicated that the deficiency depended on the homozygous condition of the recessive allele of a single gene. Mortality in the complement deficient guinea pigs was significantly higher following infection by certain pathogens (Rich, 1923; Hyde, 1923).

Variations of genotype in mice permits separation of strains which are resistant, susceptible, or of intermediate resistance to infection by S. typhimurium. Gowen (1960) found that the response of these mice to typhoid infection was an inherited characteristic and could not be attributed to acquired immunity. The experiments further suggested that response to immunization with heat killed S. typhimurium resulted in 100- to 200-fold increase of resistance in these strains. The relative differences of resistance, after vaccination, however, remained unchanged, paralleling those found in the natural resistance and dependent on the genotype of the host.

Lurie and co-workers (1952) demonstrated in inbred rabbit strains varying resistance to infection by bovine or human

tubercle bacilli, which was genetically controlled. The acquired resistance that followed infection or immunization with attenuated tubercle bacilli was greater in the strains which possessed natural resistance than that in the susceptible strains. The determinants of natural resistance were found to be multiple, complex and additive in nature.

In man, susceptibility to certain infectious agents may be attributable to genetic factors. Congenital agammaglobulinemia is a hereditary disorder occurring predominantly in male children and usually transmitted as a sex-linked recessive characteristic (Gitlin, Janeway, Apt, and Craig, 1959). (The deficiency in plasma globulins in this disease is due to an almost complete failure to synthesize gammaglobulins and respond with production of specific antibodies after infection or antigenic stimulation.) (The affected individuals have very low resistance to infections caused by gram-positive bacteria but are not unusually susceptible to infections caused by gram-negative organisms and seem to have, to some degree, the ability to develop immunity to certain viral diseases.) Isohemagglutinins are generally absent or present in very low titer. Despite the deficiency in gammaglobulins and antibodies in this disorder, hypersensitivity reactions of the delayed type are not infrequent and may be artificially induced (Porter, 1956).

Nutrition Malnutrition may cause a reduction of resistance to infection. In general, susceptibility to infection may depend on the physiologic condition of the host. Helweg-Larsen and co-workers (1952) gave an account of health problems which affected internees in German concentration camps. Among the infectious disease most frequently occurring were the ones caused by microorganisms endemic in the normal European communities. Exotic, unusual epidemices like typhus, cholera or even bacillary dysentery were uncommon. In the opinion of the authors, increase in person-to-person contact could hardly account for the aggravation of these endemic diseases. The loss of natural resistance caused by malnutrition and other forms of physiologic misery seemed far more important. Most of the internees overcame disease due to microbial origin shortly after return to normal environment and frequently without the help of specific therapy. In the case of tuberculosis, rapid recovery usually occurred, even though no antimicrobial agent was available for its treatment.

It has been shown in experiments with exactly-defined diets that pantothenic acid deficiency can evoke latent Corynebacteria infections and also render normal animals more susceptible to experimental infection with these microorganisms (Zucker and Zucker, 1954). Axelrod and Pruzanski (1955) induced pantothenic acid or pyridoxine deficiency in

rats by feeding them specialized diets. The experiments demonstrated a marked impairment of ability of these rats to produce antibodies to a soluble protein antigen, diphtheria toxoid, or to a cellular antigen, sheep erythrocytes.

Physical and chemical Antibody formation and other forms of immune response can be affected by a number of physical and chemical factors. The effects of such factors on the different phases of the immune response are not all the same (Henriksen, 1970).

Roentgen rays and other ionizing radiation are active factors. High doses of whole body irradiation can abolish all immunologic responsiveness in an animal. Radiation inhibits antibody synthesis by destruction of small lymphocytes, and its mechanism is undoubtedly the depletion of these radio-sensitive cells (Schwartz, 1971). Also, cortisone and related corticosteroids reduce antibody formation by selectively suppressing small lymphocytes and eosinophils (Henriksen, 1970).

Other immunosuppressive substances include: (1) alkylating-agents whose effect resembles that of radiation, i.e., denaturation of protein and DNA, (2) purine analogues, which block interconversion of nucleotides and disturb DNA synthesis, (3) pyrimidine analogues whose incorporation into DNA renders it nonfunctional and incapable of mitosis, and (4) folic acid analogues which prevent transformation of folic

acid to folinic acid and thereby prevents the biosynthesis of purines, pyrimidines and certain amino acids (Henriksen, 1970).

As with all physiologic mechanisms, the immunologic response may be viewed as an adaptive system in which the body attempts to maintain homeostasis of the internal body environment with the external environment.

Following confrontation of the host with a foreign stimulus (antigen), there is a period of dysequilibrium. Immunologic balance is then restored by an appropriate immunologic response. A perturbation occurs if the stimulus and the response are inappropriate to each other. Any derangement in homeostasis would result in production of undesirable sequelae thus interfering with immunologic responses.

OBJECTIVES

The objectives of the experimentation outlined in this dissertation were: (1) to determine the amounts of lead that produce subclinical effects in mice when injected in soluble form (2) to determine the effect of this heavy metal on resistance of mice to infectious disease and (3) to determine immunologic mechanisms whereby lead increases the susceptibility of mice to bacterial infection.

MATERIALS AND METHODS

Experimental Animals

Young adult female white mice (Swiss-Webster strain) were used in all experiments. The mice were of uniform size and age, weighing 18-20 grams and were procured from a commercial source.[1] Approximately 1,200 mice were used in the experimentation described herein. Five mice were held in each polycarbonate cage. They were fed Purina Laboratory Chow[2] and received water ad libitum.

Lead Compounds

Reagent grade lead nitrate crystals were purchased from a commercial source.[3] Lead stock solutions for injection were prepared by dissolving 50 mg of $Pb(NO_3)_2$ in 50 ml of sterile normal saline solution (0.85% NaCl) (NSS). Twenty parts of the soluble lead stock solution were then mixed with 80 parts of NSS to prepare a 100 µg lead concentration in a 0.5 ml volume. A 250 µg lead concentration in each 0.5 ml volume was prepared similarly by mixing equal volumes of the lead stock solution and NSS. The lead solutions were sterilized by autoclaving for 10 minutes at a temperature of 240°F. and

[1]Midwest Animal Colony, Corning, Iowa.

[2]Ralston Purina Company, Brunswick, New Jersey.

[3]Allied Chemical, General Division, Morristown, New Jersey.

allowed to cool at room temperature prior to injection.

Lead Treatment of Mice

The mice used in these experiments were given daily intraperitoneal injections of either soluble lead nitrate, sodium nitrate, or NSS for 28 to 30 days. Lead solutions, as prepared above, had been previously given to normal mice for 30 consecutive days. No signs of clinical toxicity were observed in the groups of mice receiving lead as compared to control animals which received only sterile NSS. The injections of lead solutions and NSS were given in 0.5 ml amounts.

Collection and Storage of Mouse Sera

Blood from the lead-treated and control mice was collected either by decapitation of the animals or by obtaining a specimen of blood from the periorbital sinus using 100 lamba disposable capillary pipettes. In the latter technique, one-tenth milliliter aliquots of blood were mixed with 0.4 ml of NSS and allowed to clot. The diluted blood was centrifuged at 1500 g for 10 minutes and the supernatant fluid containing the serum was recovered. The dilution of serum was considered to be approximately 1:5.

In the former technique, lead-treated and control mice were anesthetized with ether and exsanguinated by decapitation.

Generally, 0.5 to 0.8 ml of blood was obtained from each animal and allowed to clot in 60 x 15 mm plastic petri dishes.[1] For some experiments, blood from a group of mice was pooled to yield a single serum specimen. The sera were processed and collected as described above. All sera were placed in one-dram screw-cap vials, promptly frozen and stored at -70°C. until used.

Bacterial Cultures

Salmonella typhimurium var. copenhagen (S. typhimurium) was the organism used throughout this study. The culture was obtained from J. A. Christopher, Department of Bacteriology, Iowa State University, Ames, Iowa. Typing and identification of the organism was originally performed at the National Center for Disease Control, Atlanta, Georgia. The initial culture was taken from a nutrient agar slant maintained at 4°C, and all further cultures used were made from slant cultures of the original. This strain produced acid and gas from glucose, maltose, sorbitol, xylose, rhamnose, inositol and trehalose, but did not ferment lactose, sucrose or salicin. Hydrogen sulfide was produced but urea was not hydrolyzed and indole was not produced. The bacterial agent was cultured in 4 ml of trypticase soy broth for 24 hours

[1]Falcon Plastics, Div. Becton, Dickinson and Co., Oxnard, California.

at 37°C. Following the 24-hour incubation period, a standardized quantity of the culture was transferred to 4 ml of fresh trypticase soy broth and incubated at 37°C for an additional 4 hours. To check for contamination and/or mutations, colonies of the organism were routinely grown on blood agar, brilliant green and tergitol agar plates at 37°C for 24 hours. Figure 1 depicts characteristics of a 4-hour bacterial culture of S. typhimurium.

Viable Cell Count of S. typhiumurium

The viable cell population was determined using the pour plate method. The 4-hour cultures were diluted by 5-fold serial dilutions in sterile saline dilution blanks. Five-tenths milliliter of each dilution was pipetted into duplicate sterile plastic petri plates and mixed with approximately 15 ml of sterile melted plate count agar.[1] The plates were rotated gently to spread the bacterial cells, thus allowing even distribution of the bacterial suspension throughout the agar. The agar was allowed to solidify at room temperature, and the plates were incubated in an inverted position at 37°C. After 24 hours, bacterial colonies were counted and counts were averaged for each duplicate plating. The total number of organisms was determined by multiplying the number of

[1]Difco Laboratories, Inc., Detroit, Michigan.

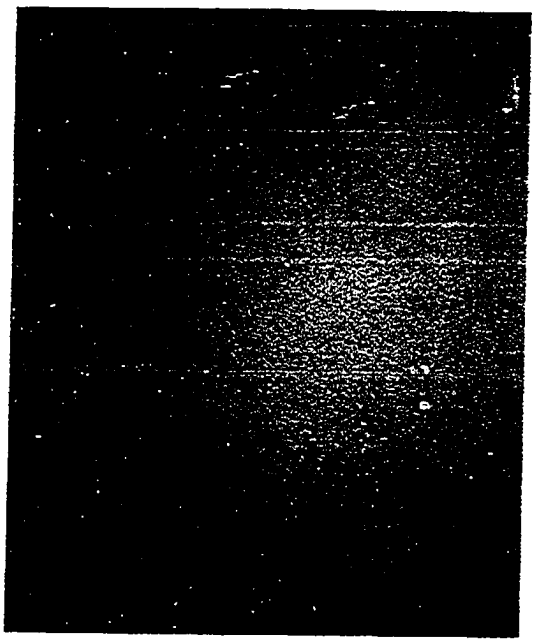
Figure 1. Photographs showing characteristics of bacterial culture of S. typhimurium var. copenhagen

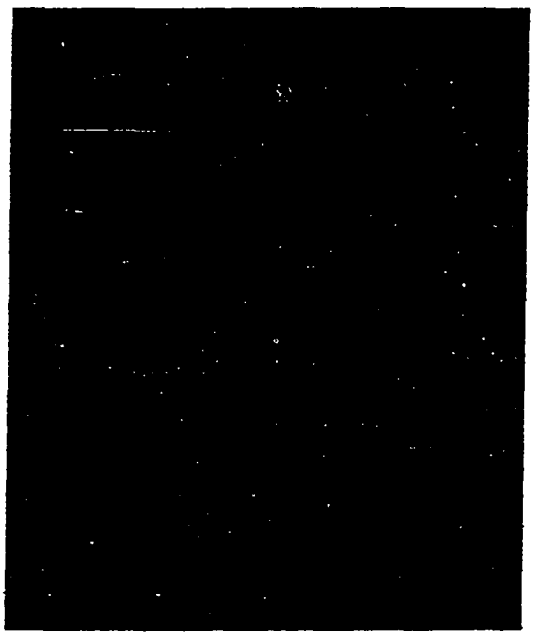
upper left - colonies of 24-hour growth on blood agar

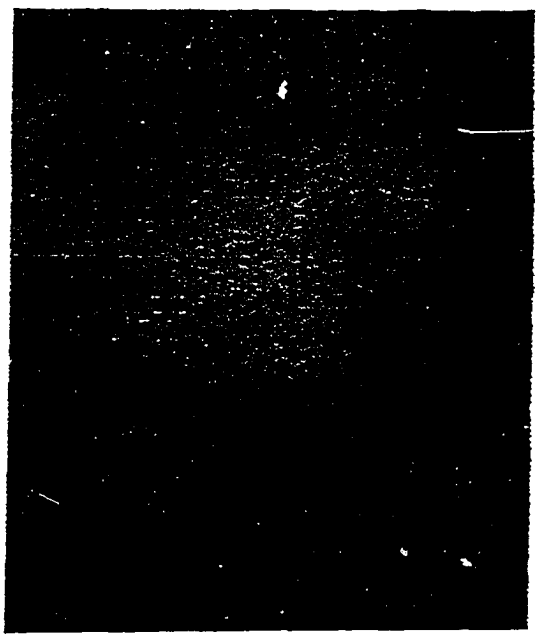
upper right- colonies of 24-hour growth on brilliant green agar

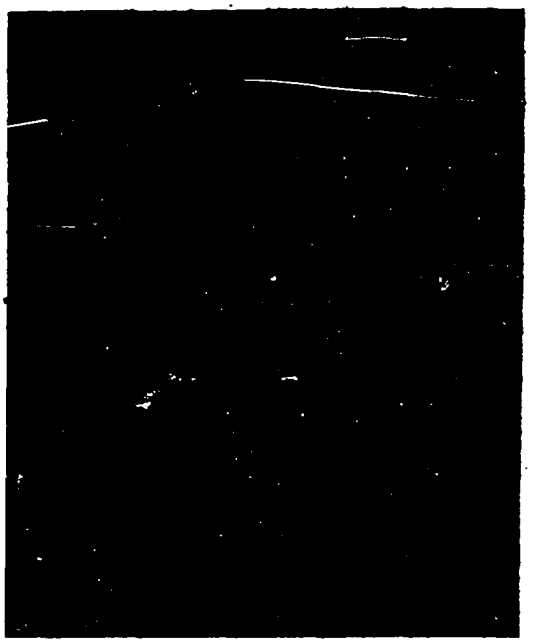
lower left - colonies of 24-hour growth on tergitol agar

lower right- specific Salmonella fluorescent antibody stain of the bacterial organism









colonies counted by the appropriate dilution factor.

LD_{50} Titration of *S. typhimurium*

A 4-hour culture of *S. typhimurium* was grown in trypticase soy broth as previously described. Five-fold serial dilutions of the undiluted culture were made in sterile saline dilution blanks. A total of five dilutions were used corresponding to 10^{-3}, $10^{-3.5}$, $10^{-4.0}$, $10^{-4.5}$, and 10^{-5}. Previous titrations of the agent indicated that a 4-hour culture contained approximately 10^{8} organisms per ml as determined by plate counts. Five mice per dilution were inoculated intraperitoneally with 0.5 ml of the bacterial suspension. The death rate of the mice was monitored for 20 days. The LD_{50} of the 4-hour culture for normal mice was determined to be $10^{-3.7}$ as calculated by the method of Reed and Meunch (1938).

Bacterial Agglutination Test

The antibody titer of each mouse serum was determined by the microtiter tube agglutination test of Takatsy (1955) in microtiter agglutination plates.[1] The reaction involved direct agglutination of thrice-washed formalin-killed *S. typhimurium* cells by the specific antibody. Reagents

[1]Cooke Engineering Co., Alexandria, Va.

used in the test were 0.85% NaCl solution, 0.1% safranin dye solution, formalin-killed S. typhimurium cells and mouse anti-S. typhimurium serum. The bacterial cells were grown in gauze-stoppered and paper-taped sterilized Ehrlenmeyer flasks containing 2 liters of sterile trypticase soy broth. They were incubated for 72 hours at 37°C with continuous moderate shaking on a variable speed rotator.[1] Following the incubation period, the cells were chemically killed by adding to each flask a volume of commercial formaldehyde solution[2] to give a final concentration of 0.35% formaldehyde. Bacteriologic assays were performed by streaking a sample of the formalin-treated cells on blood agar plates, brilliant green agar and tergitol plates at 24 and 48 hours after the addition of formalin. Sterility tests were also made by transferring a drop of the bacterial cell suspension into thioglycollate broth and incubating for 3-5 days at 37°C. The formalin-killed bacterial cell suspensions were transferred to 250-ml screw-capped centrifuge bottles and sedimented by centrifugation at 1500 g for 60 minutes in an International Centrifuge, Model UV.[3] The sedimented cells were resuspended in NSS, transferred to 50-ml screw-capped centrifuge tubes and then washed five times with 40 ml of NSS each time. The cells were

[1]Clay-Adams, Inc., New York, N.Y.

[2]Matheson, Coleman and Bell, Inc., Norwood, Ohio.

[3]International Equipment Co., Needham Heights, Mass.

stored at 4°C and were always thrice-washed with NSS just prior to use in the agglutination test. Standardization of the bacterial suspension for use in the agglutination test was made by transferring a volume (0.15 ml) of packed cells to a tube and diluting with sterile NSS to a density equal to tube 4 of a McFarland nephelometer (1200 x 10^6 bacterial cells). Enhanced visibility of the agglutination reaction was accomplished by staining the standardized suspension of bacterial cells with 0.1 ml of a 0.5% solution of Safranin O dye. The dye was added directly to a tube containing 20 ml of suspended cells. The tube was shaken, then incubated in a water bath at 37°C for 10 minutes. The stained cells were sedimented by centrifugation at 2000 g. They were next washed in NSS and then resuspended to their original volume in NSS.

Serial two-fold dilutions of the mouse serums in NSS diluent were made in microtiter plates containing 0.05 ml of diluent. The diluted serum, processed as described above, was added to the first tube without further dilution. The starting dilution was considered to be 1:5. To each tube containing 0.05 ml of the serum dilution, a drop (0.05 ml) of safranin-stained phenol-killed S. typhimurium cell suspension was added. Control tubes containing only the saline diluent plus the safranin-stained cells were included. Other controls consisting of a high-titer rabbit anti-S. typhimurium serum

and a normal nonimmune mouse serum were also included with each test as a known positive and a known negative control respectively. The plates were shaken to disperse the cells and left undisturbed at room temperature for 8 to 12 hours. A positive reaction consisted of a layer of uniformly agglutinated cells covering the bottom of the tubes. In a negative test, the bacterial cells were massed as a discreet button or a smooth ring at the bottom of the tubes. Partial agglutination was characterized by a smooth matting of cells with irregular edges. The highest serum dilution with complete or partial agglutination was considered the end point. The agglutination titer was expressed as the number of the end point tube in the series of dilutions.

Serum Protein Determination by Cellulose Acetate Electrophoresis

To ascertain the effect of lead on serum protein components, individual serum specimens corresponding to bleeding dates of 0, 4, 8, 12, 16, 20, 24 and 28 days were prepared for analysis by cellulose acetate electrophoresis. Total protein determinations were made of each serum sample by applying a drop (0.05 ml) of serum directly onto the glass window of a refractometer[1] equipped with a scale for

[1]T. S. Meter, American Optical, Buffalo, N.Y.

calibrating the total quantity of serum protein in grams/100 ml. The separation of serum protein was carried out using the Beckman microzone electrophoresis system[1] and accessory kit. The accessory kit contained blotters, Spinco B2 veronal buffer, interconnecting cable, electrophoresis membranes, fixative-dye solution, glass drying plate, plastic envelopes, sample applicator, sample covers, squeegee, 5 trays and covers and a wash bottle.

The cellulose acetate membranes were soaked with the buffer solution (pH 8.6; μ=0.075) by careful flotation followed by immersing the membranes completely to avoid trapped air bubbles which produce dry spots on the membrane thus interfering with the electrophoretic pattern. The membranes were lightly blotted between two paper blotters to eliminate excess moisture or fluid droplets. The membrane was next positioned on the connecting bars of the microzone cell in such a way that the edges of the membrane were evenly deployed within the veronal buffer filling the chambers of the cell. The cellulose acetate membranes accommodated 8 serum samples per run and the samples were applied by use of a wire applicator calibrated to deliver about 1.0 to 1.5 lambda of the sample. After checking the anode and cathode connections, the top of the apparatus was put in place and the serums

[1]Beckman Instruments, Inc., Fullerton, Calif.

were electrophoresed for 60 minutes at a constant current of 15 milliamperes and a constant voltage of 250 volts. The separated serum protein zones were stained by immersing the membrane (for 3-5 minutes) in a solution of Ponceau S stain (0.2%) in 7.5% aqueous trichloroacetic acid and 7.5% sulfosalicylic acid. Rinsing of the cellulose acetate membranes was performed by removing the membrane from the stain with forceps to allow excess stain to drain away and then immersing the membranes in several rinses of 5% acetic acid for 1 minute each. After staining and rinsing, the cellulose acetate membranes were cleared by dehydrating in 95% ethanol for 1 minute, followed by immersion for 3 minutes in a clearing fluid consisting of 10% acetic acid in 95% ethanol. The wet membrane was positioned onto a glass plate which had been previously placed in the bottom of the clearing solution tray. The glass plate and membrane were removed together from the clearing tray. Excess moisture was removed by gently but firmly drawing a squeegee over the full surface of the membrane. The membrane and glass plate were dried in an oven preheated to about 65°C for 30 minutes. When the membrane was completely dried, it could be easily lifted from the glass plate and fixed between transparent plastic sealing sheets (envelopes) which were provided in the microzone accessory kit. The fixed membranes could be scanned immediately or permanently stored in envelopes for later

scanning.

Quantitation of the relative serum protein concentrations from the stained cellulose acetate membranes was accomplished by densitometry. A Gelscan^{R} automatic recording and integrating scanner[1] was used in these studies to measure the light absorption by the stained bands of protein. Serum protein peaks were separated corresponding to albumin, alpha$_1$-globulin, alpha$_2$-globulin, beta-globulin and gamma-globulin. Automatic continuous recording of the optical density (O.D.) and continuous integration of the area under the recorded curve of the O.D. were used in calculating the percent of the total protein corresponding to each separate peak.

Whole Body Determination of Lead in Mice

Lead-treated and nonlead-treated (control) mice were used in this experiment. The mice of each treatment group received daily intraperitoneal injections of either 100 µg or 250 µg of lead nitrate for a total of 28 days. At four-day intervals, 2 mice of each treatment group and 1 mouse from the control group were euthanized and weighed on an analytical balance. Each mouse was placed into a Sorvall[2] blender con-

[1]Gelman Instrument Co., Grand Prairie, Texas.

[2]Ivan Sorvall, Inc., Norwalk, Conn.

taining 20-30 ml water and 20 mg magnesium acetate per gram of mouse (magnesium acetate is used as a catalyst). After blending, the contents were quantitatively transferred into a porcelain crucible. The crucible was placed on a steam bath until the contents became well dried, usually overnight. The crucible was then placed into a muffle furnace at a temperature of 450-475°C. At this temperature the sample was ashed within a period of 4-5 hours. When the crucible had cooled, the ash was dissolved in 2.0 N HCl, quantitatively transferred, and diluted to a 100 ml volume in a volumetric flask. A 10-ml aliquot of the sample was placed in a separatory funnel and the pH adjusted to 6.5-7 with NaOH. One milliliter of ammonium pyrolidine dithiocarbamate was added, and the sample was shaken for 1 minute. Five to 10 ml of methyl isobutyl ketone (MIBK) was added, and the sample was again shaken for 2 minutes. The two layers were allowed to separate and the aqueous layer discarded. The MIBK layer was transferred to a centrifuge tube and centrifuged at 1500 x g for 10 minutes. Disposable pipettes were used to transfer the MIBK to a clean tube. A blank and standards ranging from 0.2-2.0 parts per million (ppm) lead were extracted in a manner similar to the samples. A Perkin-Elmer Model 303 atomic absorption spectrophotometer[1] was used in the assay.

[1]Perkin-Elmer Corp., Norwalk, Conn.

The wavelength (2833A), slit width, and lamp current were those specified as standard conditions. The burner head was adjusted until the top of the burner was approximately 1/4-3/8 inch below the light path at the center of the burner. The burner was lit under normal flow conditions of air and acetylene. Water-saturated MIBK was aspirated and the acetylene flow set at 3.5 to 4.0 on the flow meter. The absorbance of the standards and samples was read using either the mil meter or a recorder.

Histopathology of Lead-Treated and Control Mice

Two mice from each of the 3 groups were killed every 4 days over a period of 28 days. Organs surgically removed for histologic examination included liver, spleen, kidney and brain. Each organ was weighed on an analytical balance and the weights were recorded corresponding to the animal from whence they came. The organs of each animal were kept together and placed in jars containing 10% buffered formalin for 48 hours. After the 48-hour fixation period, the organs were removed from formalin and transferred to jars containing 70% alcohol and stored under refrigeration at 4°C until they were paraffin embedded, sectioned and stained with hematoxylin and eosin (H & E).

Hematology

Blood samples were collected from the periorbital sinuses of lead-treated and control mice at intervals of 14, 21 and 30 days after daily intraperitoneal injections of either lead nitrate or saline. Total red blood cell and white blood cell counts were enumerated using a Coulter automatic cell counter, Model A.[1] A 0.1-ml aliquot of blood from the lead treated and control mice was mixed with 50 ml of a commercial diluting fluid (Isoton)[2] to make a 1:500 dilution of cell suspension. A second dilution equivalent to 1:50,000 was made from each 1:500 dilution by transferring 0.2 ml of the initial cell suspension into 20 ml of Isoton. Total red cell counts were made of the 1:50,000 dilution. Total counts of white blood cells were accomplished by adding 0.2 ml of a 1% saponin solution to each 1:500 dilution of suspended cells. Threshold settings on the Coulter counter for red and white blood cell enumeration were those recommended by the manufacturer.

The blood obtained for differential leukocyte counts was also collected from the periorbital sinus of lead-treated and control groups of mice. A drop of blood from each mouse was smeared on clean microscopic glass slides.

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[1]Coulter Diagnostics, Inc., Miami Springs, Fla.

[2]Coulter Diagnostics, Inc., Miami Springs, Fla.

The slides were stained with Wright's stain and 100 cells per slide were identified.

Zymosan Assay of Properdin

Symbols and nomenclature of the properdin system

P Properdin

RP Serum deficient in properdin

Z Zymosan

PZ Properin-zymosan complex

C' Complement

C'1 First component of C'

C'2 Second component of C'

C'3 Third component of C'

C'4 Fourth component of C'

R3 Serum deficient in C'3

R4 Serum deficient in C'4

Preparation of reagents

Human serum Blood from 25 healthy human donors was drawn and allowed to stand at room temperature for 2 hours. The clot was then rimmed and the blood stored at 4°C overnight. The serum was separated by centrifugation at 2500 g for 20 minutes and recentrifuged under identical conditions to remove residual red cells. The clear serum was recovered and stored at -70°C until used in the properdin assay.

Barbitol buffer solution pH 7.4

85.0 g NaCl

5.75 g 5,5-diethylbarbituric acid

3.75 g sodium, 5,5-diethylbarbiturate

5.0 g 0.15M $MgCl_2$

1.5 g 0.15M $CaCl_2$

The ingredients were dissolved in about 500 ml of hot distilled water. The solution was cooled, and distilled water then added to a final volume of 2 liters. This concentrated buffer was stored at 4°C. One part of the buffer was added to 4 parts of distilled water before use. The diluted buffer was prepared daily and discarded after a 24-hour period.

Serum deficient in properdin (RP) A sample of normal human serum was brought to and maintained at 17°C in a constant temperature bath (the control of temperature is extremely important). Temperatures below 15°C result in an incomplete removal of properdin from serum, while temperatures above 18°C result in increased loss of C'3 activity. Two milligrams of commercially prepared zymosan (Lot No. 3216E)[1] in 0.08 ml of barbital buffer pH 7.4 was added to each ml of serum. The mixture was manually stirred every ten minutes. After 1 hour, the mixture was transferred to chilled centrifuge

[1]General Biochemicals, Chagrin Falls, Ohio.

tubes and centrifuged at 3500 g for 30 minutes at 4°C. The clear supernatant fluid was brought to 17°C and 2 mg of zymosan in 0.08 ml of barbital buffer was again added with stirring to each ml of treated serum. The mixture was incubated at 17°C for 1 hour and centrifuged as before. The clear supernatant fluid (RP) was recovered and dispensed into screw-capped glass tubes for storage at -70°C or maintained at 4°C for testing within a 12-hour period.

Preparation of R3 A volume of normal human serum was added to an equal volume of barbital buffer pH 7.4. Zymosan dissolved in barbital buffer was added to the mixture at the rate of 3 mg per ml of normal serum and suspended evenly in the serum. The mixture was next incubated at 37°C for 60 minutes, with periodic manual mixing. After the incubation period, the mixture was centrifuged at 3000 g for 30 minutes at 4°C. The clear supernatant fluid was transferred to screw-capped glass tubes for storage at -70°C or held at 4°C for use within a period of 12 hours.

Standardization of R3 R3 was tested: (a) for lytic activity against sensitized sheep red blood cells; (b) for anticomplementary properties against small amounts of fresh serum; and (c) for its ability to measure C'3 in a standard RP in the presence or absence of zymosan and in the presence or absence of properdin. One-tenth milliliter of R3 should be

nonlytic for 1 ml of sensitized sheep red cells. Five one-hundredths milliliter of R3 should measure at least: (a) 120 units of C'3 per ml of RP, (b) not less than 90 units per ml of RP previously treated with zymosan at 37°C and (c) no C'3 in RP previously treated with zymosan in the presence of 1 unit of properdin per ml of RP.

Anti-sheep erythrocyte rabbit serum Glycerinated anti-sheep hemolysin (amboceptor) was obtained from Baltimore Biological Laboratory, Baltimore, Maryland.

Sensitized sheep erythrocytes Fresh sheep blood was collected in an equal volume of sterile Alsever's solution (Bukantz et al., 1946) and maintained at 4°C. The blood was stored for 4 days before use and discarded after 10 days. Sensitized sheep cells were prepared daily, as follows: cells were washed twice with 10 volumes of 0.15 M NaCl and then once with 10 volumes of barbital buffer pH 7.4. A 2% suspension of washed cells was then made in barbital buffer. One milliliter of this suspension was hemolyzed by the addition of 24 ml of distilled water. The hemoglobin concentration of the hemolyzed red cells was determined in a Beckman spectrophotometer[1] at a wavelength of 541 mμ with a violet filter using a water blank. The red cell suspension was diluted with barbital buffer to contain approximately 5×10^5 erythrocytes per ml of cell suspension by reference to a standard curve

[1]Beckman Instruments, Inc., Fullerton, California.

relating cell count and O.D. at 541 mμ. An equal volume of barbital buffer, containing 4 units of amboceptor was slowly added to the standardized sheep cells with constant swirling of the contents. The mixture was then incubated in a water bath at 37°C for 15 minutes. The cells were centrifuged as before and the supernatant fluid decanted. The cells were resuspended in barbital buffer and diluted to approximately 1.5% when used. The sensitized cells were kept refrigerated at 4°C and were usually used within a 12-hour period after their preparation.

Conduct of the assay

The method of assay for properdin depends upon the requirement for properdin for the inactivation of C'3 by zymosan as described by Pillemer and colleagues (1954, 1956). Since properdin is not the only factor necessary for this reaction, a satisfactory reagent (RP) has to supply all of the other factors which participate in the formation of the properdin-zymosan (PZ) complex and the factor(s) necessary for the inactivation of C'3 by the PZ complex. In addition, the reagent must contain adequate amounts of C'3 and the remaining components of complement required for hemolysis of sensitized sheep red blood cells (Pillemer et al., 1956).

The assay of properdin is a two stage procedure involving determination of the smallest volume of test sample which,

when incubated for 1 hour at 37°C with constant amounts of RP and zymosan, results in complete inactivation of C'3 in the mixture. A typical protocol of initial reaction mixtures as used in the experiments described herein is given in Table 2.

Table 2. Typical protocol for titration of properdin

Tube No.	RP	Test Sample	Zymosan Suspension	Barbital Buffer
1	0.1 ml	0.1 ml	1 mg	To a
2	0.1	0.05	1 mg	final
3	0.1	0.025	1 mg	volume
4	0.1	0.01	1 mg	of 0.3 ml
5	0.1	0.01 (1:2 dilution)	1 mg	
Control				
6	0.1	0	1 mg	To a final
7	0.1	0	0	volume of 0.3 ml

EXPERIMENTAL PROCEDURES AND RESULTS

Effect of Lead Treatment on Mouse Resistance to S. typhimurium

Three groups of 25 mice each were formed and housed five per cage. Each mouse was given a daily injection of either soluble lead nitrate or NSS intraperitoneally (IP) for 30 days according to the following paradigm:

Group I - 100 µg lead nitrate in 0.5 ml saline solution

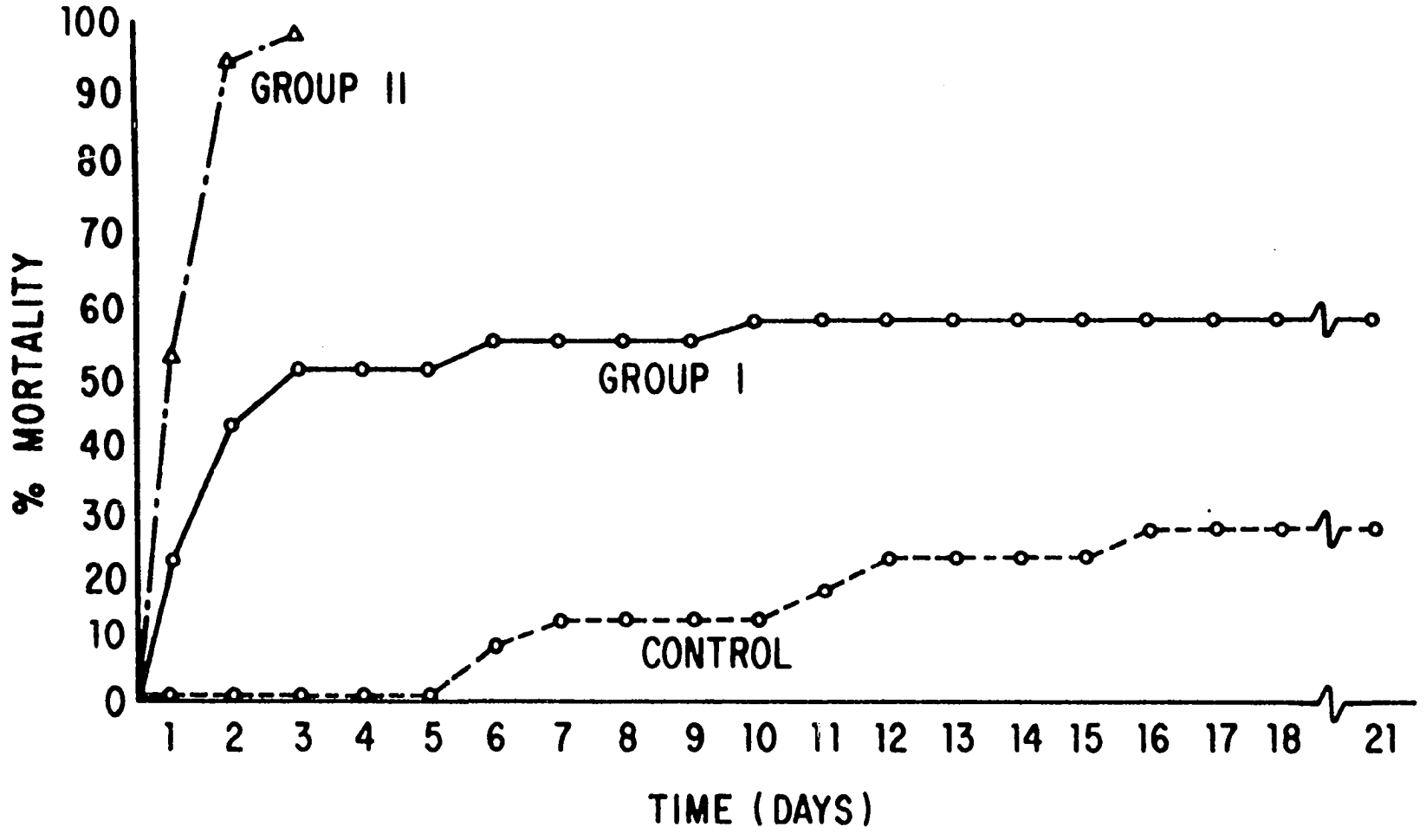
Group II - 250 µg lead nitrate in 0.5 saline solution

Group III- 0.5 ml sterile saline solution

A 4-hour culture of S. typhimurium which had previously been shown to have an LD_{50} of $10^{-3.7}$ was used as the challenge agent. The culture contained approximately 10^8 organisms per ml as determined by plate counts. Each group of five mice was challenged 24 hours after the final injection of lead or saline with a dilution ($10^{-3.0}$, $10^{-3.7}$, $10^{-4.0}$, $10^{-4.7}$, or $10^{-5.0}$) of a 4-hour culture of S. typhimurium. Mice were observed daily for signs of illness and mortality. Confirmation of death by S. typhimurium was made by necropsy and culture of organs from the dead mice.

The effect of lead treatment on mouse resistance to bacterial challenge is shown in Figure 2. Following challenge with S. typhimurium, 54% of mice in Group I but only 13% of the control group died within 7 days. All of the mice in

Figure 2. A graph showing the accumulated mortality of three groups of mice treated for 30-days with daily injections of $Pb(NO_3)_2$ or normal saline solution (NSS) and subsequently challenged with S. typhimurium var. Copenhagen. Treatments were as follows: Group I - 100 µg $Pb(NO_3)_2$ per day, Group II - 250 µg $Pb(NO_3)_2$ per day and Group III - sterile NSS


100
90
80
70
60
50
40
30
20
10
0
% MORTALITY
GROUP II
GROUP I
CONTROL
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 21
TIME (DAYS)

Group II, which had received 2-1/2 times more lead nitrate than Group I, died by the third day after challenge. At termination of the experiment, the mortality was highest in mice challenged with the lowest dilutions of bacterial culture. The calculated LD_{50} for S. typhimurium in the control group was $10^{-3.7}$ while that in Group I was $10^{-4.7}$. An LD_{50} for the mice in Group II could not be determined since all mice died.

A statistical analysis was performed to compare the mortality of mice in the three treatment groups during the first 24 hours after challenge. All mice in a group irrespective of a dilution of the challenge agent were pooled. Table 3 summarizes the statistical comparison between the various groups of mice. A chi-square (χ^2) test indicated a highly significant difference ($P<0.01$) between the lead-treated and control groups. There was also a highly significant difference ($P<0.01$) between Groups I and II which received 100 µg and 250 µg of lead nitrate, respectively.

To demonstrate that suppressed resistance in $Pb(NO_3)_2$-treated mice was actually due to lead and not to nitrate, mice were again assigned to three groups; two of which received concentrations of sodium nitrate calculated to be equivalent to the nitrate content of the lead nitrate solutions. The third group of mice served as controls and received only sterile NSS. All groups of mice received daily intraperitoneal

Table 3. Statistical comparisons of Salmonella typhimurium challenge in mice exposed to lead nitrate

Group and Treatment	Group Comparisons	χ^2 Values
I 100 µg Lead Nitrate	Group III vs. Group I and II	(P<0.01)
II 250 µg Lead Nitrate	Group I vs. Group II	(P<0.01)
III Saline Controls		

injections for 30 days and were challenged with a dilution of viable S. typhimurium as previously described. The mice were observed for mortality for 18 days. The mortality in the sodium nitrate-treated groups was no greater than the mortality in the controls.

Immune Response of Lead-Treated and Control Mice to S. Typhimurium Bacterin

One-hundred-three mice were used in this study. The mice were assigned to four groups. Group I contained 28 mice and the remaining groups contained 25 mice each. Group I received 0.5 ml daily intraperitoneal injections of 100 µg of soluble lead nitrate for 14 days. On days 15 and 17, the mice received intraperitoneal injections of S. typhimurium cells which had previously been killed in 0.35% formaldehyde solution and resuspended in sterile NSS. The bacterial sus-

pension (bacterin) was given in 0.2 ml amounts and contained approximately 2.4 x 10^8 bacterial cells as estimated by a McFarland standard. Group II mice were treated similarly to Group I except that each mouse received 250 µg of lead nitrate intraperitoneally in a 0.5-ml dose. After the second bacterin injection, both groups were continued on daily intraperitoneal injections of soluble lead nitrate for an additional two-week period.

Groups III and IV served as controls for the two lead-treated groups. Mice in these two groups received daily injections of 0.5 ml of sterile NSS intraperitoneally following the same regimen as described for Groups I and II. Group III received the two injections of S. typhimurium bacterin on days 15 and 17 and the mice of both control groups were continued on sterile NSS for an additional two weeks.

The state of immunity was ascertained by the presence or absence of antibody in blood obtained from the periorbital sinus as determined by an agglutination test. The number of mice per group, the treatment of the various groups, and the sequence of treatments are summarized in Table 4 and the results of the experiment are shown in Table 5. More than 80% of the mice in the lead-treated groups (I and II) responded with serum antibody formation following injection of the S. typhimurium bacterin. Ninety-six percent of the immunized controls (Group III) also responded to the bacterin prepara-

Table 4. Protocol for treatment of lead-treated and control groups of mice

Days	Treatment[a]	Groups I	II	III	IV
1-14	100 µg $Pb(NO_3)_2$ (0.5 ml)	+[b]	-[c]	-	-
	250 µg $Pb(NO_3)_2$ (0.5 ml)	-	+	-	-
	NSS (0.5 ml)	-	-	+	+
15	Agglutination test	+	+	+	+
	Salmonella bacterin (0.2 ml)	+	+	+	-
16	$Pb(NO_3)_2$ and NSS as above (0.5 ml)	+	+	+	+
17	Salmonella bacterin (0.2 ml)	+	+	+	-
18-20	$Pb(NO_3)_2$ and NSS treatments continued daily	+	+	+	+
21	Agglutination test (4 days)	+	+	+	+
22-28	$Pb(NO_3)_2$ and NSS continued daily	+	+	+	+
30	Agglutination test	+	+	+	+
	Number of mice	28	25	25	25

[a]All injections were given by intraperitoneal route.

[b]Treatment received or test performed.

[c]Treatment described was not done.

Table 5. Antibody response of lead-treated and control mice to a S. typhimurium bacterin. The mice were injected with bacterin 15 and 17 days following initiation of lead-treatment and antibody titers were determined 14 days after administration of the bacterin

Group	No. of Responders/ No. in group	% Responders	Range of Agglutination tube titers	Mean tube titer of: Responders	Mean tube titer of: Group
I 100 g $Pb(NO_3)_2$ and S. typhimurium bacterin	24/28	86	1-6	2.5	2.1
II 250 g $Pb(NO_3)_2$ and S. typhimurium bacterin	20/25	80	1-5	2.3	1.9
III Sterile NSS and S. typhimurium bacterin	24/25	96	2-8	6.8	6.5
IV Sterile NSS only	0/25	-	-	-	-

tion; these mice had received no lead. In contrast, none of the mice in Group IV which had received only sterile saline throughout the 30-day exposure period showed an immune response as determined by the agglutination test.

The mean tube titer of the bacterin-immunized controls was much greater than that of either of the lead-treated groups (I and II). However, the mice which had received 250 µg of lead showed no greater reduction in their mean tube titer than did the mice in Group I which had received only 100 µg of lead.

To determine whether or not the antibody responses of the three groups were significantly different from one another, the results were statistically analyzed using the Student's "t" test.

In the calculation, the antibody titers were expressed as end-point tube numbers. The dilutions started at 1:5 and the last tube with complete agglutination was considered the end-point. For example, an antibody titer of 1:160 was expressed as tube 6.

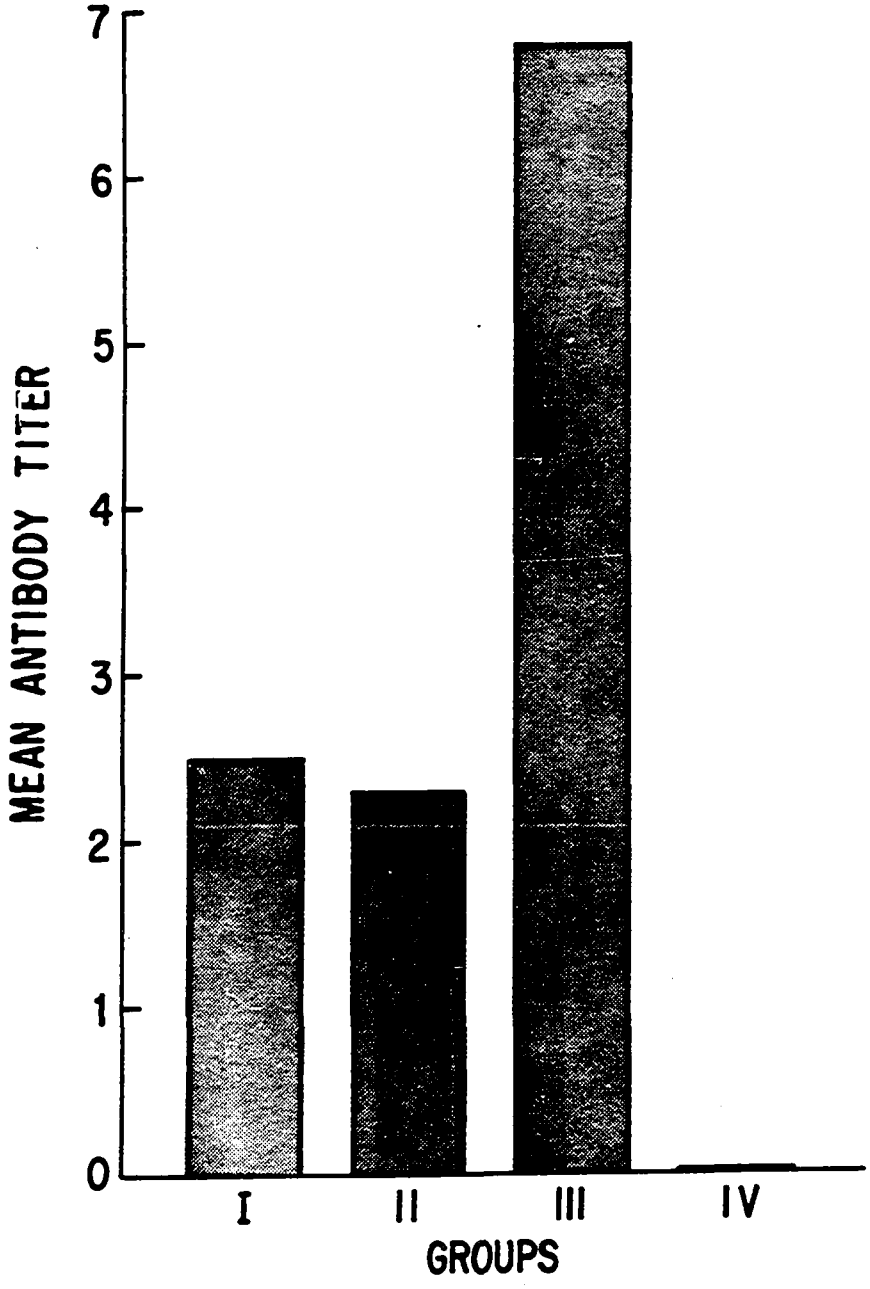
Table 6 summarizes the statistical comparison between the various groups of mice. No significant difference was observed between Groups I and II (100 and 250 µg of lead nitrate respectively) since their mean response to the S. typhimurium bacterin was essentially the same. When the mean tube titers of either Group I or Group II were compared

Table 6. Statistical comparisons of lead-treated and control mice 14 days after intraperitoneal injections of a S. typhimurium bacterin

Group and Treatment	Group Comparisons	P Values
I 100 µg $Pb(NO_3)_2$ and S. typhimurium bacterin	I vs II	No significant difference
II 250 µg $Pb(NO_3)_2$ and S. typhimurium bacterin	I vs III	(P<.001)
III Sterile NSS and S. typhimurium bacterin	II vs III	(P<.001)
IV Sterile NSS only	III vs IV	(P<.001)

with the mean tube titer of Group III, there was a highly significant difference (P<.001). There was also an expected significant difference between the response of Group IV and all of the other groups since this group received only sterile NSS and served as a normal negative control group. Figure 3 graphically compares the serum antibody titrations by the agglutination test.

Figure 3. Mean antibody titers of lead-treated or control groups of mice in response to a bacterial injection of formalin-killed S. typhimurium cells suspended in sterile 0.85% NaCl solution. A dose of bacterin contained approximately 2.4 x 10^8 bacterial cells in a 0.2 ml volume. The mean antibody titer of each group was determined by the bacterial agglutination test conducted on serum obtained 14 days following immunization


MEAN ANTIBODY TITER
7
6
5
4
3
2
1
0
I
II
III
IV
GROUPS

Analysis of Serum Protein Components by Cellulose Acetate Electrophoresis

Serum immunoglobulin patterns determined by electrophoresis on cellulose acetate membranes showed that the mean values for the alpha$_1$ (α_1) and gamma (γ) serum components of mice receiving lead were significantly reduced as compared to normal controls. There was no appreciable differences between the remaining protein concentrations of lead-treated and control groups of mouse serums. Tables 7, 8 and 9 summarize the calculated serum protein concentrations and mean values in grams per hundred ml of the three groups of mice.

As shown in the data from Tables 7, 8 and 9, there were significant differences in the γ-globulin levels between the various treatment groups. However there was also marked individual variation between mice in a particular group. These differences are reflected in the patterns pictured in Figures 4, 5 and 6.

Whole Body and Organ Weight Determinations of Lead-Treated and Control Mice

A total of 132 mice were used for determining total lead content of the mice. In this study, mice were randomly assigned to one of three groups:

1. A group which was given 100 µg of lead nitrate daily for 30 days.

Figure 4. Comparative electrophoretic patterns of representative serums from mice of Group I

upper left - serum protein pattern of a mouse serum 8 days following initiation of lead treatment

upper right- serum protein pattern of a mouse serum 12 days following initiation of lead treatment

lower left - serum protein pattern of a mouse serum 16 days following initiation of lead treatment

lower right- serum protein pattern of a mouse serum 24 days following initiation of lead treatment

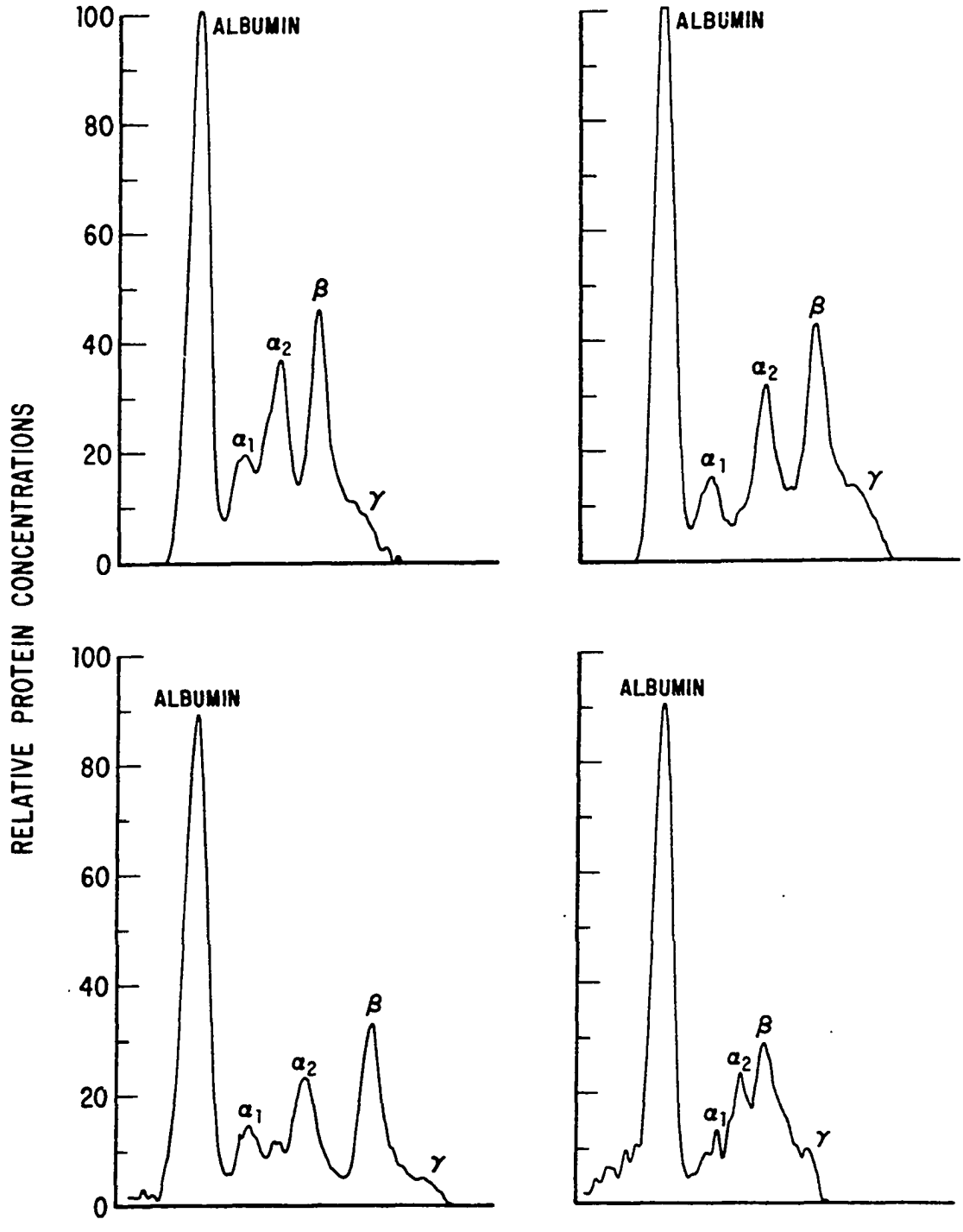

RELATIVE PROTEIN CONCENTRATIONS
100
80
60
40
20
0
ALBUMIN
α1
α2
β
γ
ALBUMIN
α1
α2
β
γ
ALBUMIN
α1
α2
β
γ
ALBUMIN
α1
α2
β
γ

Figure 5. Comparative electrophoretic patterns of representative serums from mice of Group II

upper left - serum protein pattern of a mouse serum 8 days following initiation of lead treatment

upper right- serum protein pattern of a mouse serum 12 days following initiation of lead treatment

lower left - serum protein pattern of a mouse serum 16 days following initiation of lead treatment

lower right- serum protein pattern of a mouse serum 24 days following initiation of lead treatment

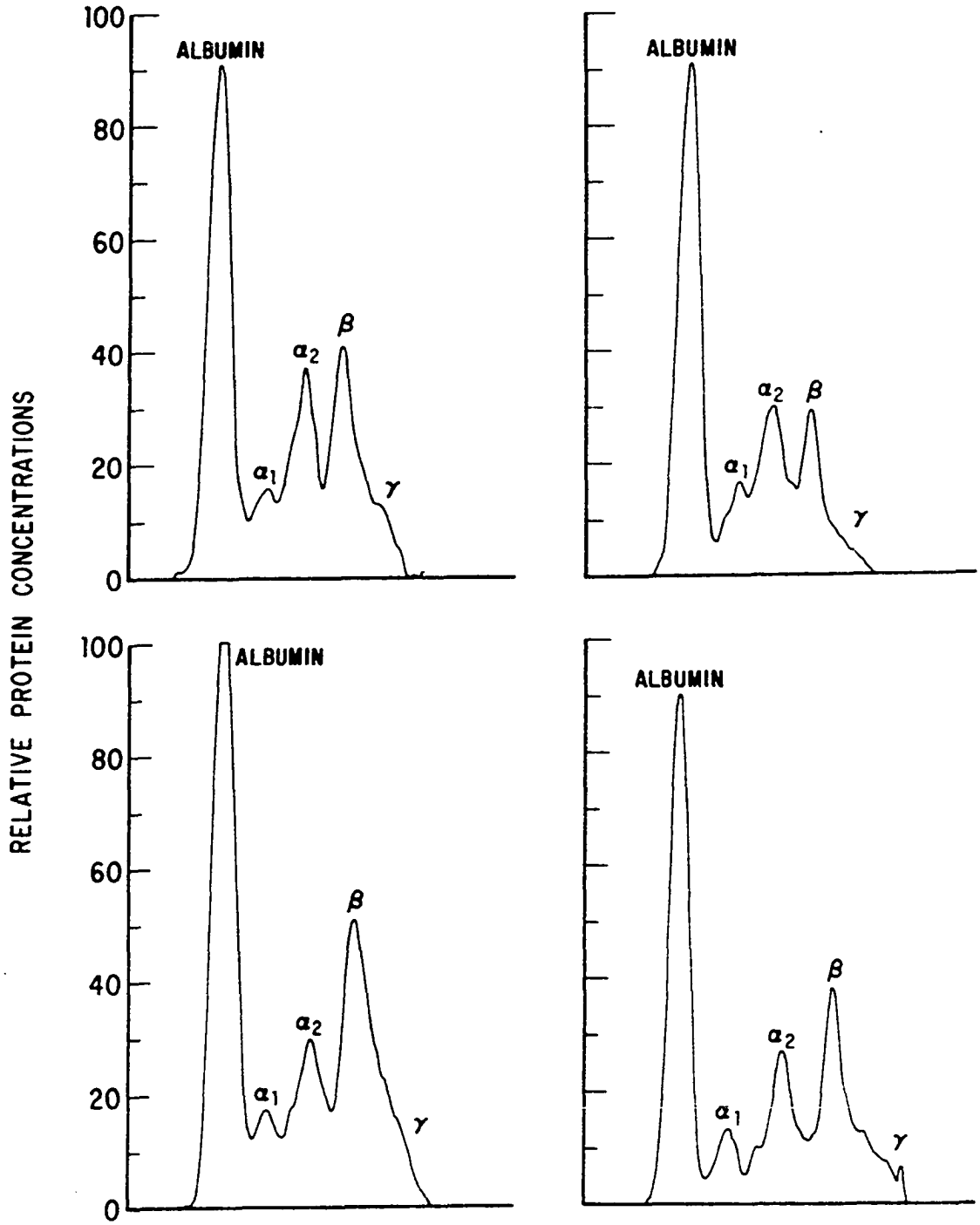

RELATIVE PROTEIN CONCENTRATIONS
100
80
60
40
20
0
ALBUMIN
α_1
α_2
β
γ
ALBUMIN
α_1
α_2
β
γ
ALBUMIN
α_1
α_2
β
γ
ALBUMIN
α_1
α_2
β
γ

Figure 6. Comparative electrophoretic patterns of representative serums from control mice in Group III

upper left - serum protein pattern of a mouse serum 8 days following daily injections of sterile normal saline solution (NSS)

upper right- serum protein pattern of a mouse serum 12 days following daily injections of sterile NSS

lower left - serum protein pattern of a mouse serum 16 days following daily injections of sterile NSS

lower right- serum protein pattern of a mouse serum 24 days following daily injections of sterile NSS

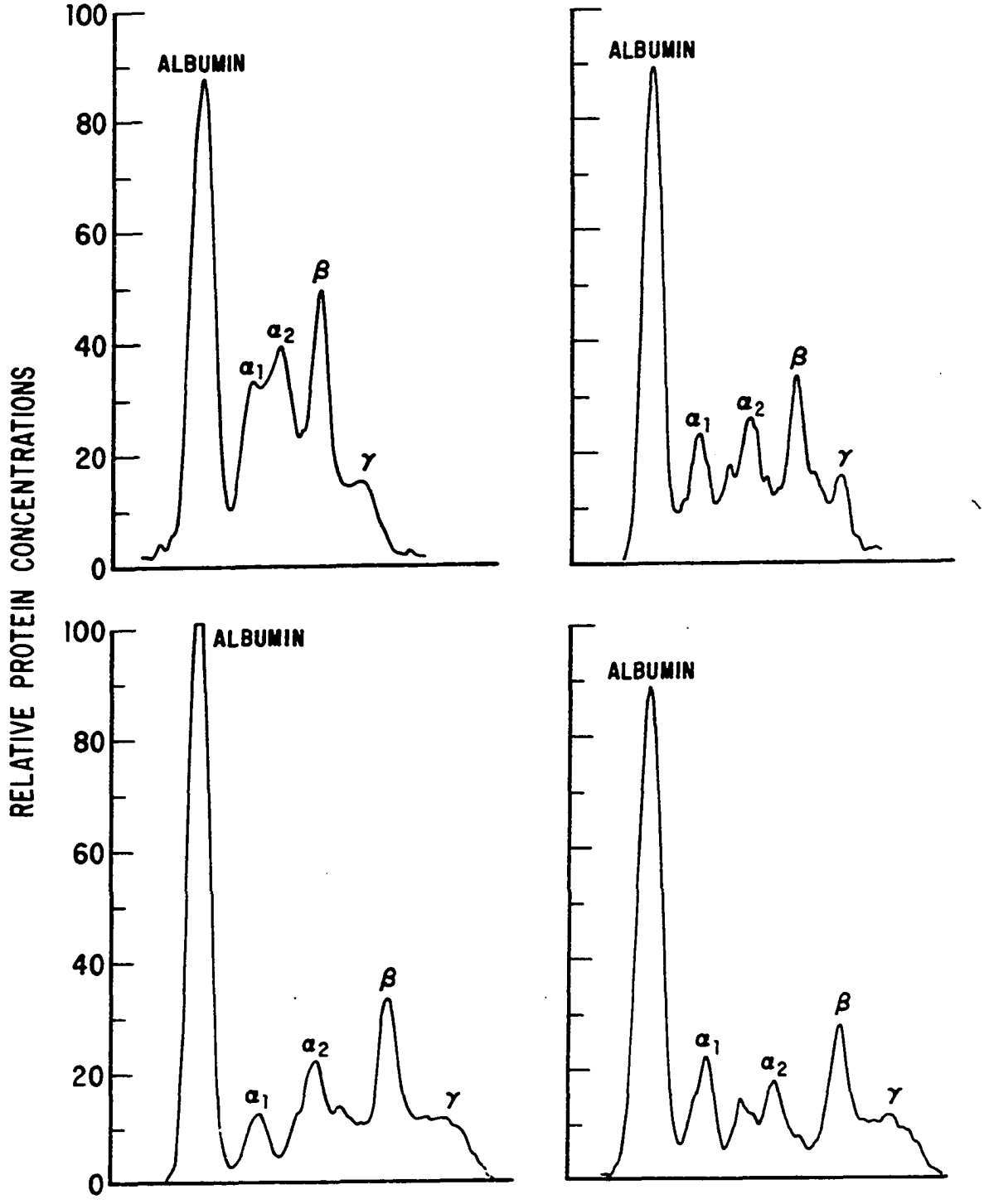

RELATIVE PROTEIN CONCENTRATIONS
100
80
60
40
20
0
ALBUMIN
α1
α2
β
γ
ALBUMIN
α1
α2
β
γ
ALBUMIN
α1
α2
β
γ
ALBUMIN
α1
α2
β
γ

Table 7. Electrophoretic distribution of serums from lead-treated mice in Group I as determined by cellulose acetate electrophoresis

Group I 100 µg $Pb(NO_3)_2$	Day[a]	Total Protein	Grams Protein/100 ml Serum[b]				
			Albumin	α1	α2	β	γ
	4	5.62	1.8	.36	1.4	1.1	.31
	8	5.83	2.6	.45	1.1	1.2	.22
	12	4.90	2.7	.43	1.0	1.5	.37
	16	6.10	2.9	.66	1.1	1.1	.42
	20	5.84	2.7	.43	1.1	1.2	.14
	24	6.00	3.0	.51	1.1	1.4	.12
	28	5.86	3.6	.46	.8	1.4	.19
Mean		5.71	2.7	.47	1.1	1.2	.25

[a]Individual mice were exanguinated on day indicated following lead-treatment.

[b]Quantitation of serum components were determined by cellulose acetate electrophoresis.

Table 8. Electrophoretic distribution of serums from lead-treated mice in Group II as determined by cellulose acetate electrophoresis

Group II 250 µg $Pb(NO_3)_2$	Day[a]	Total Protein	Grams Protein/100 ml Serum[b]				
			Albumin	α1	α2	β	γ
	4	5.48	2.1	.52	1.3	1.5	.35
	8	4.25	2.3	.30	.9	1.1	.20
	12	6.21	3.7	.48	1.4	.9	.21
	16	5.36	3.5	.35	1.1	1.4	.29
	20	5.84	2.6	.39	.9	1.8	.23
	24	4.90	2.2	.50	1.1	1.5	.28
	28	5.48	2.7	.43	1.2	1.5	.28
Mean		5.35	2.6	.43	1.1	1.4	.26

[a]Individual mice were exanguinated on day indicated following lead-treatment.

[b]Quantitation of serum components were determined by cellulose acetate electrophoresis.

Table 9. Electrophoretic distribution of serums from control mice in Group III as determined by cellulose acetate electrophoresis

Group III Saline Controls	Day[a]	Total Protein	Grams Protein/100 ml Serum[b]				
			Albumin	α1	α2	β	γ
	4	6.30	3.0	.51	1.1	1.2	.45
	8	5.86	2.9	.79	1.4	1.6	.57
	12	6.20	2.9	.75	1.2	1.2	.48
	16	4.85	2.6	.60	1.1	1.0	.48
	20	5.72	2.5	.40	1.1	1.1	.63
	24	5.84	3.5	.80	1.2	1.1	.52
	28	6.30	3.1	.66	1.1	1.0	.59
Mean		5.87	2.9	.64	1.2	1.2	.53

[a]Individual mice were exanguinated on day indicated following injections of sterile NSS.

[b]Quantitation of serum components were determined by cellulose acetate electrophoresis.

2. A group which was given 250 μg of lead nitrate daily for 30 days, and

3. A control group which received only sterile NSS.

Mice from each group were sacrificed on the following days of the experiment: 4, 8, 12, 16, 20, 24, and 28. Whole body weight and organ weights for brain, spleen, liver and kidney were recorded for each mouse.

The growth (body weight) values are significantly ($P<.025$) different for the three groups. Examination of the mean values in Table 10 show that the control group increased in weight during the experiment, but Groups I and II are changed very little, if at all. On the average, the body weight of the Group II mice was less than that of the mice in Group I (Table 10).

The mean values for the organ weight data are listed in Tables 11, 12, 13 and 14. Only in the case of the brain weight was there any indication of group differences. On the average, the brain weights for Groups I and II were significantly ($P<.05$) larger than the controls. For all groups brain weight tended to increase significantly ($P<.05$) during the experiment. No significant changes ($P>.10$) in the spleen weights were observed. The analysis of the liver data indicated a significant ($P<.025$) increase in liver weight during the experiment, but no difference between the groups. The analysis of the kidney data shows a significant ($P<.005$) increase in kidney

Table 10. Whole body weight determinations in lead-treated and control mice. The values were determined as mouse weight in grams at 4-day intervals following initiation of lead treatment

Group	Days							Mean
	4	8	12	16	20	24	28	
I 100 µg $Pb(NO_3)_2$	23.7	25.2	24.9	23.3	24.2	23.8	26.5	24.5
II 250 µg $Pb(NO_3)_2$	18.9	22.6	19.5	19.3	24.3	21.9	20.9	21.1
III Sterile NSS	22.6	21.5	24.9	29.1	32.3	30.2	32.5	27.6
Mean	21.7	23.1	23.1	23.9	26.9	25.3	26.7	

Table 11. Brain weight determination in lead-treated and control mice. The values were determined as brain weight in grams of 4-day intervals following initiation of lead treatment

Group	Days							Mean
	4	8	12	16	20	24	28	
I 100 µg $Pb(NO_3)_2$	0.39	0.43	0.44	0.41	0.44	0.46	0.39	.43
II 250 µg $Pb(NO_3)_2$	0.34	0.39	0.43	0.40	0.42	0.40	0.43	.40
III Sterile NSS	0.37	0.34	0.39	0.33	0.42	0.40	0.41	.38
Mean	.37	.39	.42	.38	.43	.42	.41	

Table 12. Spleen weight determination in lead-treated and control mice. The values were determined as spleen weight in grams at 4-day intervals following initiation of lead treatment

Group	Days							Mean
	4	8	12	16	20	24	28	
I 100 µg $Pb(NO_3)_2$	0.09	0.10	0.22	0.14	0.12	0.26	0.20	.16
II 250 µg $Pb(NO_3)_2$	0.11	0.13	0.24	0.16	0.12	0.21	0.16	.16
III Sterile NSS	0.11	0.13	0.11	0.11	0.12	0.11	0.14	.12
Mean	.10	.12	.19	.13	.12	.19	.17	

Table 13. Liver weight determination in lead-treated and control mice. The values were determined as brain weight in grams at 4-day intervals following initiation of lead treatment

Group	Days							Mean
	4	8	12	16	20	24	28	
I 100 µg $Pb(NO_3)_2$	1.15	1.41	1.62	1.43	1.58	1.80	1.41	1.49
II 250 µg $Pb(NO_3)_2$	1.13	1.14	1.57	1.24	1.60	1.73	1.52	1.42
III Sterile NSS	1.05	1.32	1.31	1.05	1.58	1.51	1.39	1.32
Mean	1.11	1.29	1.50	1.24	1.59	1.68	1.44	

Table 14. Kidney weight determination in lead-treated and control mice. The values were determined as kidney weight in grams at 4-day intervals following initiation of lead treatment

Group	Days							Mean
	4	8	12	16	20	24	28	
I 100 µg $Pb(NO_3)_2$	0.29	0.36	0.39	0.39	0.37	0.39	0.35	.36
II 250 µg $Pb(NO_3)_2$	0.22	0.34	0.37	0.34	0.39	0.42	0.36	.35
III Sterile NSS	0.27	0.27	0.33	0.31	0.42	0.38	0.39	.34
Mean	.26	.32	.36	.35	.39	.39	.36	

weight during the experiment but no difference between the groups. There is a suggestion of no further kidney growth towards the end of the experiment.

Lead Retention Studies in Lead-Treated and Control Mice

Mice from the previous experiment were utilized for determinations of whole-body lead content. The quantity of lead in each animal was calculated in parts per million (PPM) as measured by atomic absorption spectrophotometry. Appreciable quantities of lead were retained in the tissues of the mice as shown in Table 15. From examination of the mean values in Table 11 and application of analysis of variance it is apparent that the level of lead did not change over time in the control group. Both lead-treated groups had a significant ($P<.001$) increase in the level of lead during the course of the experiment. The analysis suggests that the time response may not be the same for these two groups. However, the high level in Group II at the 16th day, the low value at the 28th day and the two missing values make it difficult to interpret the results. There is no doubt, that on the average, the level is significantly ($P<.001$) greater for Group II than Group I, but it is not clear just how the difference changes over time.

Table 15. Whole body lead determination in lead-treated and control groups of mice. The values were determined as parts per million (PPM) of lead at 4-day intervals following initiation of lead treatment

Group	Days							Mean
	4	8	12	16	20	24	28	
I 100 µg $Pb(NO_3)_2$	-[a]	15.8	16.3	34.3	25.2	40.2	56.2	29.0
II 250 µg $Pb(NO_3)_2$	35.7	37.8	64.6	83.5	79.1	-[a]	69.4	62.6
III Sterile NSS	0.9	1.1	0.8	1.1	1.6	1.1	0.4	1.0
Mean	17.4	18.2	27.2	39.6	35.3	36.5	41.9	

[a]Samples were lost.

Gross and Histopathologic Changes in Lead-Treated Mice

Gross and histologic examination of brain, liver, spleen and kidney tissues from lead-treated mice revealed no pathologic changes.

Hematology of Lead-Treated and Control Mice

An experiment was conducted in which the same three experimental groups were set up and RBC and WBC determinations were made at the following times after the start of lead treatment:

1. 14 days: bled prior to 1st bacterin injection
2. 21 days: bled post 1st bacterin injection, and
3. 30 days: bled post 2nd bacterin injection

The animals were injected with a killed suspension of S. typhimurium as described in the section "Materials and Methods."

The analysis of the RBC data and examination of the mean values in Tables 16, 17, 18 and 19 show that there was a significant ($P<.001$) decrease in the RBC level during the experiment for the two groups receiving lead but not for the control group. The analysis of the WBC data (Tables 16, 17 and 18) showed a significant ($P<.01$) increase in WBC level from the beginning to the end of the experiment but there was no significant difference between the mean values of the

Table 16. Total RBC and WBC values of mice in Groups I at 14, 21 and 30 days following initiation of treatment with 100 µg/day of $Pb(NO_3)_2$

Mouse Number	14 days		21 days		30 days	
	RBC $x10^6$/cmm	WBC	RBC $x10^6$/cmm	WBC	RBC $x10^6$/cmm	WBC
21	7.84	9,900	8.07	16,400	8.92	26,700
22	10.14	5,300	7.95	7,650	7.62	8,500
23	9.06	9,600	8.74	17,000	7.62	15,000
24	9.45	12,300	8.45	15,500	7.80	10,400
25	8.74	9,700	7.23	7,700	7.03	12,800
26	8.25	12,300	8.07	22,600	7.67	7,300
27	8.13	7,900	8.20	11,400	7.27	Spilled
28	9.00	10,300	7.88	11,900	8.20	17,500
29	8.51	8,739	8.13	9,050	7.92	18,500
30	8.53	11,800	8.97	16,400	7.49	21,400
Total	87.65	97,830	81.69	135,600	77.54	138,100
Mean	8.76	9,783	8.17	13,560	7.75	15,344

Table 17. Total RBC and WBC values of mice in Group II at 14, 21 and 30 days following initiation of treatment with 250 µg/day of $Pb(NO_3)_2$

Mouse Number	14 days		21 days		30 days	
	RBC $x10^6$/cmm	WBC	RBC $x10^6$/ccm	WBC	RBC $x10^6$/ccm	WBC
31	9.94	9,600	6.45	7,200	8.07	16,400
32	10.14	10,900	7.91	7,700	8.45	15,500
33	9.32	16,300	8.05	18,700	7.23	7,700
34	9.47	13,700	8.13	6,400	8.07	22,600
35	8.88	13,100	8.30	11,900	8.20	11,400
36	8.49	17,300	6.51	20,100	7.91	7,700
37	8.51	17,300	7.81	21,100	8.05	18,700
38	9.07	21,000	7.08	19,500	6.51	20,100
39	8.13	7,050	9.79	12,300	7.81	21,100
40	8.49	16,600	8.56	15,300	7.08	19,500
Total	90.44	142,850	78.59	140,200	77.38	160,700
Mean	9.04	14,285	7.86	14,020	7.74	16,070

Table 18. Total RBC and WBC values of mice in Group III at 14, 21 and 30 days following daily injections of sterile NSS

Mouse Number	14 days		21 days		30 days	
	RBC $x10^6$/cmm	WBC	RBC $x10^6$/cmm	WBC	RBC $x10^6$/cmm	WBC
56	9.14	7,250	8.66	11,400	7.81	13,700
57	8.02	9,450	7.31	11,000	9.02	10,300
58	8.51	19,500	8.78	13,800	8.12	15,800
59	9.44	13,300	8.75	11,500	8.46	18,300
60	9.04	9,875	7.81	11,200	8.15	19,900
61	8.84	9,000	7.96	12,100	10.09	13,400
62	6.31	5,500	8.08	7,800	7.90	19,400
Total	59.30	73,925	57.35	78,800	59.55	110,800
Mean	8.48	10,561	8.19	11,257	8.51	15,828

Table 19. Summary table of mean, total RBC and WBC values in lead-treated and control mice at 14, 21 and 30 days following initiation of treatment with $Pb(NO_3)_2$

Group	Treatment	14 days	21 days	30 days	Mean
		Red Blood Cells x 10^6/cmm			
I	100 µg $Pb(NO_3)_2$	8.76	8.17	7.75	8.23
II	250 µg $Pb(NO_3)_2$	9.04	7.86	7.74	8.21
III	Sterile NSS	8.48	8.19	8.51	8.39
Mean		8.79	8.06	7.94	8.27
		White Blood Cells/cmm			
I	100 µg $Pb(NO_3)_2$	9,783	13,560	15,120	13,046
II	250 µg $Pb(NO_3)_2$	14,285	14,020	16,070	14,792
III	Sterile NSS	10,561	11,257	15,829	12,549
Mean		11,652	13,133	15,656	13,583

three groups (Table 19).

Examination of peripheral leukocyte counts were made at time intervals corresponding to sampling periods used in the agglutination and total cell count experiment. Five mice from each lead-treated group and 2 mice from the saline controls were bled on days 14, 21 and 30. It was hoped that some evidence could be found to indicate a typical response in relation to lead treatment or bacterial immunization. Some investigators have found a lymphocytosis response, while others a polymorphonuclear leukocyte response as a result of various immunizations (Shechmeister, Paulissen and Yunker, 1956; and Campbell, 1969). In the present work the difference in the differential cell counts seems to be due to fluctuations in the lymphocyte and neutrophile leukocyte populations. The changes include both depressions and increases over the lymphocyte-neutrophile counts as indicated by control animals (Tables 20, 21 and 22).

Zymosan Assay of Properdin in Lead-Treated and Control Mice

A total of 66 mice was used in the properdin experiments. The mice were assigned to three groups as follows:

Group I - consisted of 21 mice and received daily intraperitoneal injections of 100 µg of lead nitrate in 0.5-ml amounts for 28 days.

Table 20. Differential leukocyte determinations of lead-treated and control mice 14 days following initiation of treatment

Mouse Number	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
I 100 µg $Pb(NO_3)_2$					
1	48	50	2	0	0
2	26	68	6	0	0
3	38	61	1	0	0
4	41	57	2	0	0
5	39	58	3	0	0
II 250 µg $Pb(NO_3)_2$					
1	26	74	0	0	0
2	11	88	1	0	0
3	46	54	0	0	0
4	45	49	6	0	0
5	36	60	4	0	0
III Saline Controls					
1	33	62	5	0	0
2	37	62	1	0	0

Table 21. Differential leukocyte determinations of lead-treated and control mice 21 days following initiation of treatment

Mouse Number	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
I 100 µg $Pb(NO_3)_2$					
1	27	71	2	0	0
2	28	69	3	0	0
3	40	58	2	0	0
4	19	78	3	0	0
5	31	65	4	0	0
II 250 µg $Pb(NO_3)_2$					
1	39	60	1	0	0
2	40	59	0	1	0
3	25	73	2	0	0
4	33	65	2	0	0
5	44	55	1	0	0
III Saline Controls					
1	21	78	1	0	0
2	24	76	0	0	0

Table 22. Differential leukocyte determinations of lead-treated and control mice 30 days following initiation of treatment

Mouse Number	Neutrophils	Lymphcytes	Monocytes	Eosinophils	Basophils
I 100 µg $Pb(NO_3)_2$					
1	28	72	0	0	0
2	36	57	1	0	0
3	31	68	1	0	0
4	27	72	1	0	0
5	39	61	0	0	0
II 250 µg $Pb(NO_3)_2$					
1	17	79	4	0	0
2	23	77	0	0	0
3	43	56	1	0	0
4	29	68	3	0	0
5	25	72	3	0	0
III Saline Controls					
1	28	71	1	0	0
2	31	68	1	0	0

Group II - consisted of 21 mice and received 250 µg daily doses of lead for 28 days.

Group III - consisted of 24 mice and served as controls. Mice of this group received only sterile NSS in 0.5-ml amounts for 28 days. Starting at day 0 for the control group and every 4 days thereafter for all groups, 3 mice from each group were exsanguinated by decapitation under ether anesthesia. Generally, 0.5 to 0.8 ml of blood was obtained from each animal and blood from each group of 3 mice was pooled to yield a single serum specimen for properdin titration. The serums were frozen promptly and held at -70°C.

Properdin Titration

Serum samples were thawed at room temperature. An aliquot of each test sample was diluted 1:4 in barbital buffer pH 7.4. Decreasing volumes of the initial dilutions were used to make the reaction mixtures containing RP, zymosan, buffer and test sample. The mixtures were incubated for 1 hour at 37°C and then were centrifuged at 1500 g for 10 minutes at room temperature. After centrifugation the residues were discarded and the supernatant fluids tested for C'3. Results of the properdin titration for the 3 groups of mice are shown in Table 23.

The number of units of properdin in the test samples was calculated from the least volume which would give complete

Table 23. Properdin titers of lead-treated and control mouse serums

Test Sample	Day Bled	Group I (100 µg Lead Nitrate)	Group II (250 µg Lead Nitrate)	Group III (Saline Controls)
		(units/ml)		
1	0	ND[a]	ND	≥12[b]
2	4	0	0	16
3	8	0	0	16
4	12	0	0	16
5	16	<6[c]	8	≥24
6	20	<10	≥12	≤12
7	24	≥12	<6	≥12
8	28	8	6	≥12

[a] ND = not done.

[b] ≥ equal to or greater than.

[c] ≤ equal to or less than.

inactivation of C'3 or less than 50% hemolysis in 1 ml of RP. For example, if 0.05 ml of test sample was found to be the least volume giving no detectable C'3 (complete inactivation) when incubated with sensitized sheep red blood cells and R3, then 0.05 ml of test sample has completely inactivated the C'3 in 0.1 ml of RP, or 0.5 ml of test sample should completely inactivate the C'3 in 1 ml of RP. The properdin titer is the reciprocal of this volume; 1/0.5; that is: 2 units/ml. Since the test samples were diluted 1:4 the properdin titer then becomes 2 x 4 = 8 units/ml. Extrapolations for properdin units were made on test serums which showed partial inactivation of C'3 or less than 50% hemolytic activity.

Control tubes were always set up in parallel with the test samples and carried throughout the remainder of all procedures. Control tubes were labelled A, B and C. Tube A contained 0.1 ml RP + 0.1 ml buffer + 0.1 ml zymosan. This tube was used to determine the loss of C'3 in the presence of zymosan but in the absence of properdin. Tube B contained 0.1 ml RP + 0.2 ml buffer. This control determined the concentration of C'3 in the RP after incubation, centrifugation, etc., but in the absence of properdin and zymosan. Tube C contained 1 ml sensitized sheep red blood cells and 0.3 ml buffer. This tube served only as a control on the red cells. Typical results of the control tubes gave the following

approximate values:

Tube A - >30% hemolysis

Tube B - >40% hemolysis

Tube C - No hemolysis

Since the endpoint in the properdin titration is that volume of the sample which, in conjunction with RP and zymosan, results in inactivation of all of the C'3 in the reaction mixture, it was not necessary to titrate C'3 but merely to determine whether it was present or absent. Thus, it was sufficient to test 0.2 ml of each supernatant for C'3 by adding 0.1 ml of R3 and 1 ml of sensitized sheep cells. The tubes for C'3 determination were incubated for 30 minutes at 37°C and then centrifuged at 1500 g for 5 minutes at room temperature. The supernatant fluids were inspected for hemolysis.

Absence of hemolysis meant complete inactivation of C'3 in the RP. As previously stated, it was not necessary in these studies to titrate C'3 but merely to determine its presence or absence by the use of amboceptor-sensitized sheep red blood cells. Thus, after incubation of the reaction mixtures at 37°C for 60 minutes, it was sufficient to merely test the supernatant fluids for C'3. However, a complete C'3 titration was always done on the control samples A and B to insure that the RP had a satisfactory C'3 titer, both in the presence and absence of zymosan.

It should be noted that within 4 days after administration of lead in both lead-treated groups of mouse serums, properdin activity was completely absent through day 12, while serum from the control mice had a properdin titer which ranged from 12 to 24 units/ml throughout the experiment. It is also seen that on day 16, properdin was demonstrable in both lead-treated groups of mouse serums and remained present throughout day 28. With the exception of day 20 for the 250 µg lead-treated group and day 24 for the 100 µg group, properdin titers for both treated groups were decidedly lower than the normal values in control serums. Because all of the test serums had initially been diluted 1:4 to provide more volume, the question arose as to whether properdin in the lead-treated serums was indeed completely absent as a result of lead treatment or merely reduced and, therefore, not detectable due to the dilution factor. Thus, the assay was repeated using undiluted test samples of all serums from day 4 through day 12. The results of this test were identical to those of the diluted samples. Greater than 50% hemolysis was found in all dilutions of the test samples of both lead-treated groups, whereas, properdin titers of control serum were within normal limits. This percent hemolysis indicates that properdin was not available in the serum of lead-treated mice to inactivate C'3 in the reagent for properdin (RP). Conversely, C'3 in a standard amount of RP was completely in-

activated by properdin in the test samples of the control serums at various levels of dilution. A complete titration of properdin levels of normal serum as performed in this experimentation is shown in Tables 24, 25, and 26.

Table 24. Properdin titration of normal mouse serums

RP (ml)	Test Sample (# Day Bled)	Volume ml (dilution)	Barbitol Buffer	Zymosan (mg)	R3 (ml)	Sensitized Sheep Cells (ml)	% Hemolysis	Properdin units/ml
0.1		0.1 (1:4)	-	0.1	0.1	1.0	0	
0.1	#1	0.05	0.050	0.1	0.1	1.0	0	
0.1	Day 0	0.025	0.075	0.1	0.1	1.0	15	$\geq$12
0.1		0.01	0.090	0.1	0.1	1.0	>50	
0.1		0.1 (1:4)	-	0.1	0.1	1.0	0	
0.1	#2	0.05	0.050	0.1	0.1	1.0	0	
0.1	Day 4	0.025	0.075	0.1	0.1	1.0	0	16
0.1		0.01	0.090	0.1	0.1	1.0	>50	
0.1		0.1 (1:4)	-	0.1	0.1	1.0	0	
0.1	#3	0.05	0.050	0.1	0.1	1.0	0	
0.1	Day 8	0.025	0.075	0.1	0.1	1.0	0	16
0.1		0.01	0.090	0.1	0.1	1.0	>50	

Table 25. Properdin titration of normal mouse serums

RP (ml)	Test Sample (# Day Bled)	Volume ml (dilution)	Barbitol Buffer	Zymosan (mg)	R3 (ml)	Sensitized Sheep Cells (ml)	% Hemolysis	Properdin units/ml
0.1		0.1 (1:4)	-	0.1	0.1	1.0	0	
0.1	#4	0.05	0.050	0.1	0.1	1.0	0	
0.1	Day 12	0.025	0.075	0.1	0.1	1.0	0	16
0.1		0.01	0.090	0.1	0.1	1.0	>50	
0.1		0.1 (1:4)	-	0.1	0.1	1.0	0	
0.1	#5	0.05	0.050	0.1	0.1	1.0	0	
0.1	Day 16	0.025	0.075	0.1	0.1	1.0	0	
0.1		0.01	0.090	0.1	0.1	1.0	10	$\geq$24
0.1		0.1 (1:4)	-	0.1	0.1	1.0	0	
0.1	#6	0.05	0.050	0.1	0.1	1.0	0	
0.1	Day 20	0.025	0.075	0.0	0.1	1.0	30	$\leq$12
0.1		0.01	0.090	0.1	0.1	1.0	>50	

Table 26. Properdin titration of normal mouse serums

RP (ml)	Test Sample (# Day Bled)	Volume ml (dilution)	Barbitol Buffer	Zymosan (mg)	R3 (ml)	Sensitized Sheep Cells (ml)	% Hemolysis	Properdin units/ml
0.1		0.1 (1:4)	-	0.1	0.1	1.0	0	
0.1	#7	0.05	0.050	0.1	0.1	1.0	0	
0.1	Day 24	0.025	0.075	0.1	0.1	1.0	10	$\geq$12
0.1		0.01	0.090	0.1	0.1	1.0	>50	
0.1		0.1 (1:4)	-	0.1	0.1	1.0	0	
0.1	#8	0.05	0.050	0.1	0.1	1.0	0	
0.1	Day 28	0.025	0.075	0.1	0.1	1.0	15	$\geq$12
0.1		0.01	0.090	0.1	0.1	1.0	>50	
	Test Controls							
0.1	Tube A	-	0.1	0.1	0.1	1.0	>30	
0.1	Tube B	-	0.2	-	0.1	1.0	>40	
-	Tube C	-	0.3	-	-	1.0	0	

Table 27. Electrophoretic distribution of serums from AKR and Swiss-Webster mice

Serum Samples	Total Protein g/100 ml	Relative Percentages of Serum Components				
		γ	β	α2	α1	Albumin
Normal AKR Serum	5.36	9.0	24.2	6.3	8.8	48.5
AF_c[a] Serum	4.98	7.4	33.5	17.1	7.5	30.5
Normal S-W Serum	6.6	8.0	18	18	9.6	44.3
100 µg $Pb(NO_3)_2$	5.93	4.3	21.3	18.1	7.8	46.8
250 µg $Pb(NO_3)_2$	5.90	4.4	23.8	19.3	7.1	43.6

[a] AF_c - AKR mouse immunized with complete Freunds adjuvant.

DISCUSSION

The increased susceptibility to bacterial infection in the absence of clinically observable lead toxicity would be of paramount public health significance. A large portion of our human and animal population is constantly exposed to appreciable quantities of lead due to the presence of this element in air, water and food. Lead has long been suspected of causing significant changes in normal physiological mechanisms at some point short of clinical disease. The study described herein was designed to determine the influence of subclinical lead exposure on resistance of mice to bacterial infection.

A significant aspect of this study was to determine non-lethal levels of lead over a 30-day period of treatment and to determine the effect of such treatment on the mice. During the 30-day period of exposure to "subclinical" amounts of lead, none of the mice showed toxic manifestations. Five mice died during the exposure period from other causes: one in Group I, two in Group II, and two in the control group. Figure 7 shows the healthy appearance of lead-treated and control groups of mice 28 days following initiation of the various treatments.

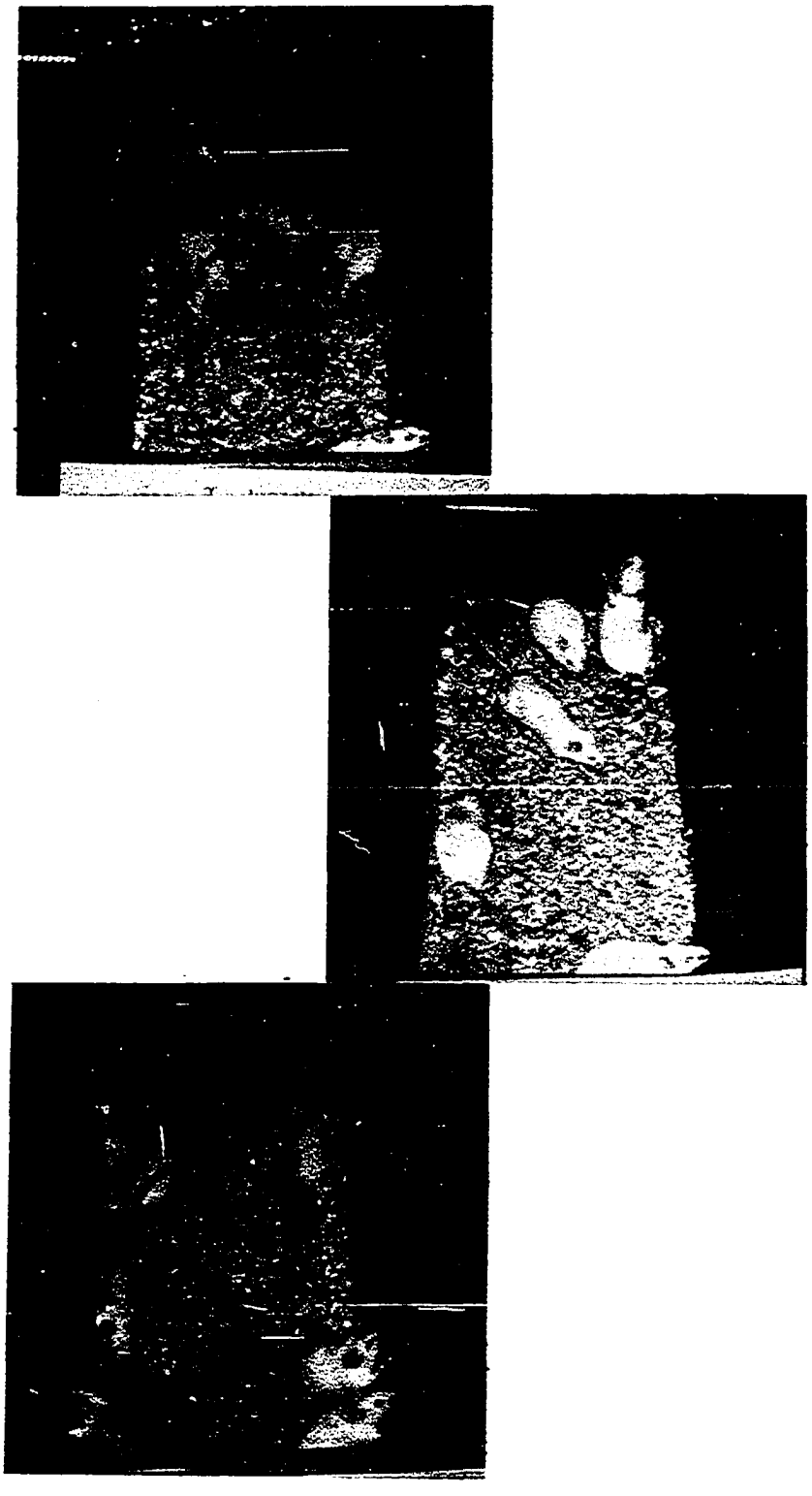
Whole body digestion of lead-treated mice to measure lead retention revealed significant concentrations of lead in

Figure 7. Photographs of lead-treated and control mice 28 days following initiation of treatment

top - mice of Group I that received daily injections of 100 µg of $Pb(NO_3)_2$

middle - mice of Group II that received 250 µg of $Pb(NO_3)_2$

bottom - control mice of group III that received only sterile NSS



the body. These concentrations appeared cumulative and increased with successive doses of lead nitrate solutions. The increased concentrations were also dose-related since more than twice as much lead was recovered from mice of the 250 µg group than from mice in the 100 µg group at any given time throughout the sampling period. Lead absorption from the peritoneal cavity was probably quite rapid, with very slow excretion and considerable retention of lead by the mice. No attempt was made in these studies to evaluate lead excretion either in the urine or feces. However, the relatively large quantities of lead recovered by the whole body digestion technique suggests that an imbalance between lead-intake and lead excretion was indeed present. Such an imbalance and the inability for the mice to rapidly eliminate small doses of lead over a prolonged exposure period would explain the dangers of what Patterson (1965) has referred to as "chronic lead insult". The cumulative action of lead, therefore, could eventually result in altered physiologic activity or in the production of signs of lead intoxication.

In spite of the fact that the lead-treated mice appeared completely normal throughout the lead-exposure period, whole body weight determinations showed that the mice of both lead-treated groups were less than those of mice in the control group. It appears also that whole body weight of each lead-treated group was related to the dose of lead administered

since the weight of mice that received daily injections of 250 µg (Group II) of lead was significantly less than that of mice in group I which received daily injections of 100 µg of lead. Since these mice were immature at the outset of the experiment, control animals gained appreciably in body weight during the treatment period in contrast to minimal growth in lead-treated animals. This observation could indicate that exposure to lead, even at subclinical levels, effectively impaired certain metabolic functions in the susceptible hosts. Similar observations have been reported for rats; Goyer et al. (1970) considers a decrease in rate of body-weight gain to be a sensitive indicator of the metabolic effects of lead. Possible contributing factors include decreased intestinal absorption, uncoupled oxidative phosphorylation and excessive loss of urinary amino acids. The latter two defects were clearly shown to be operative in rats receiving large doses of lead, but changes in these parameters were not discernable at the small doses at which decreased weight gain became apparent (Goyer et al. 1970).

Histologic examination of the brain, liver, spleen and kidney from lead-treated mice revealed no pathologic changes. In the absence of demonstrable histologic changes in any of the tissues examined and since direct quantitation of lead in these specific organs was not determined, no definite statements can be made regarding the concentration, or

distribution of lead in the various organs at this time. However, in view of the significant differences between whole body weight determinations of lead-treated and control mice, it was rather interesting that there were no differences of significance between the organ weights of the various groups. In fact, the mean organ weight of lead-treated mice were slightly greater than those of the controls. The observation that the growth rate of organs was not affected by the lead treatments while body growth rates were significantly less than controls might suggest that the relatively small amounts of lead as used in these experiments were either not toxic for cytoplasmic organelles or, conversely, lead may have initiated a very low grade inflammatory or edematous response which was not discernable on histologic examination.

It is well established that heavy metals can combine with a large variety of organic molecules by interaction with ligands such as sulphydryl, carboxyl, amino, imidazolyl or phosphoryl groups (Passow et al., 1961). Because of these interactions, lead may behave as an inhibitor of various enzymes involved in heme biosynthesis. It is therefore interesting that several studies have established that some important reactions involved in heme biosynthesis take place in the mitochondria (Dresdel, 1955; Sano, 1958; Eriksen, 1960). Castellino and Aloj (1969) studied the intracellular distribution of lead-210 in liver and kidney homogenates of rats. They

reported that, within a short time after injection of lead, the metal was incorporated in all intracellular fractions of liver and kidney homogenates obtained by differential centrifugation. They concluded that the direct interference by lead with some steps in heme biosynthesis (mainly occurring in the mitochondria) was probably due to an enzymatic block of Δ-aminolevulinic-dehydrase and heme synthetase. Goyer et al. (1970) described intranuclear inclusion bodies as related to lead dosage in rats. They were careful to point out that the clinical usefulness of this parameter is obviously very limited. It is possible, however, to recognize these inclusions in some cases of clinical lead poisoning.

Hematologic changes as a result of subclinical exposure to lead was not demonstrated in these experiments. Erythrocyte indices as well as leukocyte counts were within normal limits. In cases of lead poisoning an anemia may result that is usually characterized by microcytosis, hypochromia and stippling, accompanied by a variable number of reticulocytes (Leiken and Eng, 1963). There have been two views expressed as to the primary cause of anemia. Aub et al., (1926), found changes in the erythrocyte osmotic fragility after in vivo and in vitro exposure of these cells to lead, and concluded that a direct hemolytic effect on the circulating erythrocyte was important. Others have felt that lead exerts its

hematologic effect primarily upon erythrocyte precursors in the bone narrow and that the resultant defective erythrocytes are removed from the circulation by the spleen and RES in general (McFadzean and Davis, 1949; Pierre, 1952). It is highly possible that both mechanisms may be effective but anemia or erythrocyte alteration was not observed to result from subclinical exposure to lead.

In investigations of the effects of lead and its compounds on the blood cells, the amount of attention given to the effects on the red cells has been far greater than that given to the effects on the white cells. Numerous papers have appeared dealing with the red-cell anemia, poikilocytosis, polychromasia and basophilic stippling. The last mentioned phenomenon is very readily recognized, and is one which, although it occurs in other conditions than lead poisoning, is still notably characteristic of the malady, at least when present to a considerable degree. The significance of changes occurring in the white cells has been largely overlooked or neglected. That there are significant changes has even been denied (Shiels, 1950). Possibly as a result of the greater attention given to the red cells there have been considerable differences of opinion as to whether there are any definite changes in the white cells in lead poisoning,

Normal blood values for the mouse have been compiled by Schalm (1965). The differential leukocyte counts for

neutrophils, lymphocytes, monocytes and eosinophils are 26.2, 67.8, 7.5 and 2.0% respectively. In general, the differential leukocytes determinations of lead-treated and control mice in this study remained within normal limits throughout the experiment. The monocyte counts, however, were slightly lower than those cited by Schalm (1965) and only one eosinophile was demonstrable in Group II. It would appear from these results that a sustained lymphocytosis was not apparent and that there was no indication that the experimental animals were undergoing infection or showing any other atypical leukocytic responses.

Goadby (1912) stated that in the early stages of lead poisoning, more especially in acute cases, distinct leukocytosis was present, with a predominance of lymphocytes. Large mononuclear leukocytes were greatly increased in number. He further stated that a differential leukocyte count in cases of lead poisoning, which also showed the presence of basophilic granules, invariably brought to light an increase in the percentage of lymphocytes and a decrease in the number of polymorphonuclear cells, even when the total leukocyte count was not outside normal limits. In his opinion, while an estimation of the total number of red cells and of white cells was useful, it was not by any means as valuable as the differential leukocyte count and the presence of basophilic granules. While other workers reported that lead produced a

relative lymphocytosis, this finding was not strikingly demonstrated by Aub et al. (1926) in their experimentation. Pepper and Farley (1933) stated that there was little effect on the leukocytes in clinical cases.

Bell et al., (1925), in considering the effects of lead in the treatment of cancer, found slight leukocytosis with moderate doses of lead and pronounced leukocytosis and stated that slight relative eosinophilia was fairly common.

McLean (1930), in investigations of the effect of intravenous injection of colloidal lead into rabbits, found immediate leukocytosis due at first to neutrophilia; after eight hours, the numbers of lymphocytes and large mononuclear cells began to increase, and in two days reached a higher maximum than the granular cells. The lymphocytes exhibited a greater response to the lead injections than did the polymorphonuclear leukocytes.

Cantarow and Trumper (1944), after referring to a number of authors' conclusions, stated that the great majority of observations contradict these specific effects of lead upon the relative proportions of the white blood cells in the peripheral circulation in uncomplicated cases.

The scientific literature reveals many studies of the biochemical and pathological effects of lead, but only a few reports on immunoglobulin alterations associated with lead exposure. Sroczynski et al. (1964) studied the serum protein

fractions of 60 workers suffering from chronic lead poisoning. They found that 10% of the workers showed hypoproteinemia and reduction in α1 globulin. Serum proteins studied by Palmieniak and Smolik (1963) in 32 persons exposed to lead revealed a reduction in the α-globulin fraction as the main disorder noted. Soliman and his colleagues (1970) reported that of 112 sera from Egyptian workers exposed to lead, 19 showed certain biochemical changes. Eight of the 19 cases showed complete absence of the γ1-globulin fraction, while 11 cases showed a great reduction in the γ1-globulin fraction.

In the present work, electrophoretic distribution of serum components of normal Swiss-Webster (S-W) strain mice showed 5 distinct peaks. They were designated as γ, β, α1-globulin, α2-globulin and albumin. Jordan et al., (1962) electrophoretically characterized serum and ascitic fluid from highly inbred AKR mice and described 7 components of serum from normal and immunized AKR mice. They included a prealbumin component and divided the beta region into two components, β1 and β2. Table 27 shows the comparative distribution of serums from immunized and nonimmunized AKR mice used in the experiments of Jordan et al. (1962) and serums from lead-treated and control S-W mice as used in the present study. The relative percentages of the β1 and β2 components for AKR mice have been combined for simplicity in comparisons. It can be seen in Table 27 that immunization

with antigen-adjuvant mixtures markedly increased the pooled relative percentages of α2 and β-globulins in AKR mice as compared to AKR normal controls. The percentages of γ-globulin remained relatively unchanged.

In the present work, continuous exposure of soluble lead nitrate to mice in Group I and Group II did not increase the relative percentages of α2 or β-globulin components but, in contrast, caused a significant reduction of the γ-globulin components of lead-treated mice as compared with the normal saline controls. There was also some reduction of the α1 components of lead-treated mice but no change in the relative percentages of the remaining fractions. These results, however, do not contradict the findings of Jordan's group since the antigens used in their work (BSA-Freunds adjuvant mixture) would have stimulated or enhanced antibody formation. This could account for the increase in β-globulin concentration.

The nature of the antigenic stimulation in this study would not have been expected to affect electrophoretic patterns appreciably and, if at all, would be expected to increase α- and/or β-globulin concentration. However, since γ-globulin levels were reduced in lead-treated mice and β-globulin remained the same as controls, one must assume that lead was responsible for reducing γ-globulin concentrations.

As is known, some metals have the propensity to precipitate proteins, and this precipitation apparently is selective in nature (Kunkel, 1947). The γ-globulins are first affected, then other globulins in a definite order. Thus, Williams et al. (1954) postulated that if lead can precipitate γ-globulins, this action can also affect the antibody molecules since these are γ-globulins.

The results reported here appear consistent with the assumptions of Williams et al. (1954) that lead in appropriate concentrations can inactivate γ-globulins and other serum proteins of the antibody system. It can further be assumed that if lead can selectively precipitate or bind γ-globulins then the defense of the body against infectious agents would be either greatly reduced or destroyed (Williams et al., 1954).

Continuous exposure of mice to low concentrations of lead nitrate during a 30-day period produced no clinical signs of lead toxicity but did indicate enhanced susceptibility to bacterial infection. Such a conclusion was supported by the observation that there was a ten-fold difference in the LD_{50} of the S. typhimurium organisms between the control and the lead-treated mice. The susceptibility of the lead-treated mice to a strain of S. typhimurium with limited pathogenicity was markedly increased.

The very rapid mortality observed for mice in Group II

(all but one died within 24 hours) raises the question of how lead may increase susceptibility to S. typhimurium. Selye et al. (1966) reported that exposure of rats to lead markedly increased their susceptibility to bacterial endotoxin when both were given simultaneously. This enhancement effect probably does not fully explain the results of the experimentation discussed here. It is doubtful that quantities of endotoxin sufficient to kill mice could have been present in 0.5 ml of a $10^{-5.0}$ dilution of a 4-hour culture containing approximately 500 organisms. Furthermore, more than 24 hours elapsed between the last administration of lead nitrate and challenge with S. typhimurium. A more logical explanation would be that lead interfered with resistance mechanisms in the mice, thereby permitting uninhibited bacterial growth. In the mice receiving 250 µg of lead, lethal quantities of endotoxin could have been quickly produced in the animal with a resultant rapid mortality. In contrast, mice receiving 100 µg of lead were either not as susceptible to endotoxin or were able to restrict somewhat the growth of the bacterial agent. Irrespective of which mechanism was operational, there must have been a reduced resistance on the part of the exposed mice to bacterial multiplication.

The reduced resistance observed in this experimentation could have resulted from the action of lead on one or several immunologic mechanisms. Interference with phagocytic activity

of polymorphonuclear leukocytes has been reported in cases of lead toxicity in man (Rakhimova, 1968). Lead has also been reported to bind antibodies *in vitro* (Williams *et al.*, 1954) and could potentially do so under *in vivo* conditions. This ability to bind proteins could also interfere with the functional activity of the properdin system. This experimental parameter was investigated in this study and will be discussed subsequently.

The antibody response to a *S. typhimurium* bacterin as determined by a bacterial agglutination test demonstrated a marked reduction in serum antibody titers in both lead-treated groups of mice as compared to controls. This significant difference may suggest that antibodies were inactivated by lead following production or that lead interfered with antibody synthesis. Possibly lead and its compounds when adsorbed into the circulation, binds to functional molecules, thereby interfering with vital processes. It is well known that many other factors, physical as well as chemical may play a significant role in overcoming the resistance of man and animals. For example, corticoid deficiency after adrenalectomy or irradiation greatly decreases resistance against various bacterial products (Bellanti, 1971). It may be profitable, therefore, to explore the possibility that lead may act by interference with the function of the pituitary gland, adrenal gland or other metabolic phenomena.

It is conceivable that low-levels of lead in the body may act as a dispersing agent, much in the same fashion as urea, causing disruption of noncovalent forces on antibody molecules resulting in separation or unfolding of polypeptide chains of the molecule with subsequent loss of antibody function. "Antigen binding" sites or antibody "active sites" on immunoglobulin molecules may become blocked or inhibited by the action of lead thus interfering with proliferation of cells necessary for a complete immune response. Lead is probably not easily catabolized and daily doses, even at subclinical levels, may have been sufficient to overwhelm the capacity of the RES to either phagocytize it or to set in motion those factors necessary for adequate antibody synthesis and release.

The administration of low doses of lead produces alterations of major proportions in the properdin levels of mice. Properdin activity in both lead-treated groups of mice was either completely absent or significantly less than the normal properdin values demonstrated in control mice which received only sterile NSS. The absence of properdin from day 4 through day 12 of both lead-treated groups is apparently due to a direct and rapid action of lead and to the affinity that lead has for humoral factors of immunologic reactivity. On the basis of the results obtained, it may be concluded that lead, in subclinical amounts, has the ability to decrease or remove

circulating properdin in the early stages following lead treatment. Properdin levels either by synthesis or some release mechanism, is reestablished during the later stages of lead treatment.

The early absence of properdin may be caused by the formation of complexes of this natural protein with the injected lead, thereby allowing removal of properdin from the circulation. It is more difficult, however, to explain the reoccurrence of properdin titers from day 16 to termination of the experiment. Perhaps the hosts by day 16 were able to establish a physiologic balance between absorption and excretion of lead thus becoming "conditioned" to the treatments. Thereafter, they were then able to handle lead in such a way that properdin titers were either no longer affected or affected only to a limited degree. A second possibility is that the mice compensated for the loss of properdin by increased synthesis of the immunologic material. Properdin titers, however, did not fully return to normal levels observed in the controls and could account for observations in previous aspects of this work which demonstrated lead-mediated suppression of antibody formation as well as certain alterations in immunoglobulin patterns of lead-treated mice.

In this study, the absence or presence of properdin was not directly correlated with resistance to challenge with an infectious agent. It would now be indeed interesting to

challenge lead-treated mice and controls at a time when properdin levels are completely absent, that is, day 4 and day 8. Such an experiment would be necessary to demonstrate a correlation between normal properdin titers of control mice and resistance to certain infections; conversely, between absence of reduced properdin titers and susceptibility to these infections. Such experimentation is projected.

It is interesting to note that properdin titers in man are reportedly not affected by such factors as age, sex, leukocyte count, abnormal albumin/globulin ratio, or the presence of various antibodies (Lepow, 1959). Normal properdin titers have been observed in patients with agammaglobulinemia, unexplained recurrent infection, bronchiectasis, cirrhosis, acute leukemia, cortisone tuberculosis, and rheumatoid arthritis (Hinz, 1956). Representative patients in nonterminal stages of various malignancies also had normal properdin titers (Hinz, 1956), while terminal patients were found to have low properdin titers (Southam and Pillemer, 1957). Decreased properdin levels have been observed in patients with pneumococcal pneumonia, gram-negative infections (pyelonephritis, Hemophilus influenza, and bacillary dysentery) and paroxysmal nocturnal hemoglobinuria. In a few instances, it has been possible to correlate properdin titers with the clinical course of a given infection. Thus, the properdin titers of patients with pneumococcal pneumonia and meningo-

coccal meningitis fell to very low levels soon after onset of their illnesses and gradually returned to normal following appropriate therapy (Hinz, 1956). Perhaps, in the present investigation, had the animals lived and lead treatments been terminated, the properdin levels may have returned to normal values.

The studies outlined and described in this dissertation have shown that a general attribute of subclinical levels of lead exposure is the ability of lead to alter resistance mechanisms in mice. The decreased resistance is associated with alterations of several parameters of immunologic reactivity. In this sense, lead was capable of upsetting the immunologic balance (homeostasis) of exposed animals. Whatever may be the true underlying mechanism(s) of the capacity of lead to reduce resistance and alter immunologic reactivity, it is my guess that the lead treatments as administered in these studies induced what was comparable to an immunologic deficiency state. If this assumption is correct, then several major mechanisms of immunity, could have been impaired including phagocytosis and the inflammatory response (including complement and other aspects of the biologic amplification system); antibody responses and cell-mediated (delayed hypersensitivity) responses might also be impaired.

The complement system has already been described as a major biologic amplification system promoting inflammation,

phagocytosis and the effects of antibody. A deficiency state of any of its 9 components might result in abnormal inflammatory responses or an increased susceptibility to infection. Lead is known to affect certain enzymes and interestingly, the complement system comprises several enzymes. Lead, then could inhibit such enzymes and could interfere with physiologic processes thus leading to a complement-deficient state of the mice. The lack of hemolytic activity on the part of mouse complement precludes the use of that classical procedure for titration of complement in mice; therefore, one would need to investigate a complement-mediated type of reaction such as the properdin system for an evaluation of its activity.

Enzymes are also particularly important in the killing and digestion of microorganisms by phagocytic cells. Further, lead could interfere with antibody synthesis by binding with nucleic acids which may be significant in the inductive phase of antibody formation. Therefore, the lead-exposed mice were incapable of responding to the bacterial immunization as compared to the response observed in nonlead-treated, but immunized controls.

The data have been discussed in relation to an hypothesis that the evaluation of immunologic parameters in individuals of a population may be a valid procedure for establishing an adverse effect from the intake of quantities of lead that do not produce clinical illness. The results of this study appear consistent with that hypothesis.

SUMMARY

Investigations of the effect of lead on mice demonstrated that treatment of mice with daily doses of 100 μg or 250 μg of soluble $Pb(NO_3)_2$ for 28-30 days failed to elicit signs of clinical toxicity other than failure to gain weight. The mice appeared perfectly normal and there were no gross or histologic changes in tissues although appreciable quantities of lead were retained.

The experimentation demonstrated that subclinical levels of lead markedly increased susceptibility of the mice to challenge with a strain of S. typhimurium. Increased susceptibility, in the absence of overt clinical signs of lead toxicosis, undoubtedly indicated an influence of lead on immunologic mechanisms.

Studies of alterations in immunologic parameters were conducted by cellulose acetate electrophoresis of mouse serums, by measurement of the response of mice to immunization with S. typhimurium bacterin and by determination of properdin levels in lead-treated mice. The experiments demonstrated that subclinical levels of lead caused a significant reduction in α- and γ-globulin components of lead-treated mice as compared with normal nonlead-treated controls. Anti S. typhimurium antibody titers were significantly reduced in lead-treated mice as compared to controls. This result must have

reflected the ability of lead to either interfere with antibody production or to inactivate antibody. Properdin activity in lead-treated mice was either completely absent or markedly reduced.

The enhanced susceptibility or reduced resistance was possibly due to one or several of the immunologic mechanisms investigated in this study. These observations, collectively, provide support to the hypothesis that an immunologic deficient state was induced in mice by exposure to subclinical levels of lead.

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Fin.

FEH