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Pneumococcus
antibody
spleen
liver
immunoparalysis
reticulo endothelian system
I^{125}

Effect of Antibody upon Clearance of I^{125}-Labelled Pneumococci by the Spleen and Liver

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Extract

Clearance of I^{125}-labelled Type II pneumococci in immune and nonimmune rabbits was determined at 15, 30, 45 and 60 minutes after an injection of 1×10^9 organisms (fig. 1). The patterns of uptake by the spleen and liver in immune and nonimmune rabbits differed in several major respects. The overall uptake by the liver exceeded that of the spleen in control and immune animals by a factor of at least 10 at each interval examined, this being most striking in the immune group. The spleen had a consistently higher capacity for uptake per unit of weight than the liver at all time intervals examined. This effect was found in immune as well as in nonimmune animals. The type specificity of this organ uptake pattern of response was determined by challenging an immunized group with a type of organism which differed from the immunogen. The pattern of uptake resembles that of nonimmunized rabbits. Studies using passive immunization with type specific and heterologous type antisera confirmed these findings.

Speculation

The results obtained indicate more efficient function of the spleen in nonimmune clearance of these microorganisms but greater activity per unit of weight than the liver in both groups. The liver, although dependent upon type specific antibody for maximal clearance, was found to bear the total greatest burden of both immune and nonimmune clearance. Thus the capacity of the spleen for clearance of single episode of bacteremia may have considerable significance in the absence of specific antibody, in initiating a rapid local specific immune response there.

Introduction

A mechanism has been proposed to explain the unique susceptibility to acute, fatal infections in certain splenectomized infants which would be improbable at other times in life [8]. This mechanism proposes that the spleen is concerned with the phagocytosis of particulate antigens in the absence of humoral antibody. In the absence of circulating antibody, the splenectomized individual is susceptible to bacteremic episodes.

Recent experimental data have defined some of the immunological functions of the liver and spleen. Both the liver and the spleen are principal sites of localization of bacteria or other particulate materials which enter the circulation (reviewed in 3). The relative effectiveness of these organs in the clearance of bacteria and other particulate material appears to depend upon the presence and amount of circulating antibodies. For example, on a unit of weight basis the spleen of the normal mouse or rabbit has a greater capacity than

the liver for uptake of particulate material such as S^{35}-labelled conjugated sheep red blood cell stromata [12]. P^{32}-labelled *E. coli* are cleared preferentially by the spleen rather than the liver in the absence of opsonins [2]. In newborn piglets deficient in natural antibody, I^{131}-tagged *Salmonella* are found predominantly in the spleen [4]. Lastly, the titer of circulating antibody correlates with the relative uptake of tagged erythrocytes by the human liver or spleen; at low titers the spleen was relatively more efficient [13].

Thus it appears that small amounts of antibody are requisite for efficient reticuloendothelial function of the liver. The spleen, however, appears to function most effectively in the absence of such antibody.

The studies to be reported were designed to determine precisely the clearance functions of the liver and spleen in the presence and absence of specific antibody and thereby to examine the mechanism of preferential clearance by the spleen in the nonimmune animal. I^{125}-labelled encapsulated type specific pneumococci were employed to trace the relative uptake of organisms by these organs in the mature rabbit. For the whole organ, the results indicate that the spleen functions more efficiently than the liver in the clearance of these microorganisms in the absence of specific antibody. On a unit of weight basis the spleen is more efficient than the liver in clearing microorganisms both in the presence and absence of specific antibody. The liver, although dependent upon type specific antibody for maximal clearance, was found to bear the total greatest burden of both immune and nonimmune clearance. A mechanism for these clearance patterns is suggested by the data.

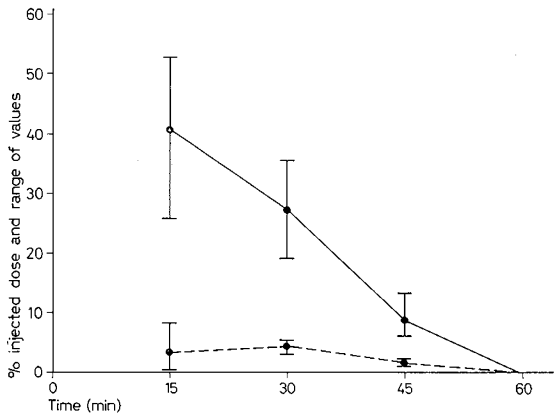

Fig. 1. Clearance of I^{125}-labelled Type II pneumococci from the circulation of the 14 normal and 14 Type II-immune rabbits after an intravenous dose of 1×10^9 organisms at time zero. No measurable activity was present in the blood of any animals 60 minutes after injection. --- = immune, —— = nonimmune.

Methods and Materials

Preparation of suspensions of pneumococci: Diplococcus pneumoniae. Type I (Strain 6301, American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md.,) and Type II (Strain A77, Communicable Disease Center, Diagnostic Reagents Section, Atlanta, Ga.[1]) were grown by a standard method [5]. Formalinized suspensions of 8-hour-old cultures were heated 20 minutes at 70° C and then washed three times in phosphate buffered saline (0.15 M, pH 7.4). Specificity of the capsular polysaccharide was verified with the use of sera obtained from Statens Seruminstitut, Copenhagen, Denmark[2]. The supernatant of the prepared suspensions contained less than 0.001 mg/ml polysaccharide, the minimum level detectable by the modification of the anthrone method described by MOKRASCH [14]. The cell suspension was adjusted to between 1.0×10^9 and 8.0×10^9 organisms/ml, and counted using a Coulter counter.

I^{125}-labelling of pneumococci. A modification of the protein-labelling technique was employed utilizing Iodine^{125} and an oxidizing agent [9]. To 2.0 ml of pneumococcal suspension, 1.0 ml of potassium iodide, in 0.15 M, pH 7.42-phosphate buffered saline was added. 0.05 to 0.1 mc of I^{125} (in $NaOH$-Na_2SO_4 solution, Nuclear Research Chemicals, Inc., Orlando, Fla.) was added and the mixture was gently shaken. Two ml of ammonium persulfate, 10 % in phosphate buffered saline, was slowly added to the reagent mixture over a one-minute interval while gently shaking the tube. The mixture was incubated at room temperature overnight and then dialyzed against phosphate buffered saline with changes of dialysate as needed until the dialysate was free of radioactivity. The supernatant of the final suspension was also free of radioactivity. The I^{125}-labelled pneumococci retained type specificity, did not appear altered morphologically, and did not show any tendency to spontaneous agglutination. Scintillation counting was used for all radioassays, employing a Nuclear Chicago model DS.202 well detector and a 181A counter.

Immunization. Adult New Zealand albino rabbits were immunized intravenously according to a modification of the method of GOODNER *et al.* [10] using pneumococci, Types I and II prepared as above.

Clearance studies. Nonimmune rabbits and rabbits previously immunized with Type II pneumococci were divided into four groups. Each animal received 1 ml

[1] Supplied by Dr. Max Moody, Communicable Disease Center, Atlanta, Ga.

[2] A gift of Dr. Tom Moore, Ross Laboratories, Columbus, Ohio.

of I^{125}-labelled Type II pneumococcal suspension (10^9 orgs/ml) in the marginal ear vein, then was anesthesized and sacrificed by bleeding at a set interval after injection. The intervals were 15, 30, 45 and 60 minutes. The percent of injected dose in 1 ml of heparinized whole blood was determined, and the total blood activity estimated by assuming a blood volume of 7 % total body weight.

Organ uptake. Nonimmune rabbits and rabbits previously immunized with Type II pneumococci were divided into four groups. Animals received an injection of 1.0 ml I^{125}-labelled Type II pneumococci (10^9 orgs/ml), and were sacrificed by bleeding at 15, 30, 45 or 60 minutes. The livers and spleens were removed at time of sacrifice, weighed, and the percent injected dose of a wet weighed aliquot of each organ was determined. The percent injected dose per gram of wet tissue and per organ was then calculated. Other groups of nonimmune rabbits received injections of 2,000 I.U. of rabbit anti-pneumococcic serum Type II or 4,000 I.U. of Type III (Lederle Laboratory, Inc.). One hour after the injection the animals were given I^{125}-labelled Type II pneumococci and organ uptake at one hour following challenge was determined as above.

The effect of immune paralysis was studied in groups of nonimmune rabbits which received intravenous injections of pneumococcal polysaccharide Type II (Lot No. 36T, Lederle Laboratories), 1 mg once a week for three consecutive weeks. One week after the last injection these and control groups of rabbits were given I^{125}-labelled pneumococci as described above, and the uptake by liver and spleen was determined.

Results

Blood clearance studies. Clearance of I^{125}-labelled Type II pneumococci in 14 immunized and 14 control rabbits was determined at 15, 30, 45 and 60 minutes after injection of 10^9 I^{125}-labelled Type II pneumococci. The results are shown in figure 1. At 15 mintues, 30 minutes and 45 minutes significant differences between the control and immune groups were found; at one hour no measurable activity was found in the blood of either the immune or the nonimmune groups. The pattern of clearance indicates that the immune animal clears the circulating blood of most organisms within 15 minutes after injection. The patterns of clearance shown do not differ substantially from those reported for other bacterial species [2, 4].

The data obtained from serum samples, collected earlier than fifteen minutes following injection of the radiolabelled organisms, were not satisfactory due to the wide variation in intravascular mixing.

Relative organ uptake of I^{125} pneumococci in immune and nonimmune rabbits. The uptake per unit of weight by the spleen and liver in early clearance of pneumococci by immune and nonimmune animals was determined in 28 nonimmune rabbits and 23 rabbits previously immunized with Type II pneumococci. These animals received intravenous injections of 10^9 I^{125}-labelled Type II pneumococci, and were sacrificed at 15, 30, 45 and 60 minutes following injection. The percent of the injected dose per gram of wet tissue and for the whole organ was determined, and a ratio comparing the uptake per unit of weight of the two tissues was calculated. The results of these experiments are shown in table I. Studies performed in the early phases of this experiment indicated that only insignificant amounts of radioactivity were detected in organs other than the liver and spleen.

The patterns of uptake by the spleen and the liver in control and immune animals differed in several major respects. The overall organ uptake by the liver exceeded that of the spleen in control and immune animals by a factor of at least 10 at each interval examined. This was most striking in the immune groups, and at the 30 minute interval this difference was over 100 fold, at which time the liver contained up to 48 % of the injected dose.

The spleen of immune animals contained one-half or less of the total organ radioactivity found in nonimmune animals at each interval examined.

Differences between liver and spleen uptake in nonimmune animals were further evaluated in terms of uptake per unit of weight. The spleen had a consistently higher capacity for uptake per unit of weight than the liver at all intervals examined. This effect was found also in immune animals in which spleen uptake per unit of weight was greater than in the liver.

Immunological specificity of organ uptake patterns. Clearance of particulate material from the reticuloendothelial system can be altered by a number of factors other than specific antibody or opsonin, including endotoxins, drugs, state of general health of the animal, and specific infection. In order to establish the immunological specificity of the differences in uptake patterns by spleen and liver, and to attribute them to the effects of specific antibody, several types of experiments were performed. In one series of experiments the clearance of I^{125}-labelled Type I pneumococci in Type II immune rabbits was examined. Results of this experiment are illustrated in table II. Immunization with Type II organisms failed to modify the response of rabbits to challenge with Type I organisms. The organ response was indistinguishable from that of nonimmune control animals.

The effect of paralysis-inducing doses of pneumococcal polysaccharide on clearance patterns was also

Table I. Uptake of I^{125}-labelled pneumococci[1, 2]

Time interval between injection and sacrifice (min)	Experimental group	Number in group	Spleen				Liver				Per gram spleen	
			Per organ		Per gram		Per organ		Per gram		Per gram liver	
15	immunized	7	2.7	±1.2	2.8[3]	±1.3	55.7	± 6.3	0.7	±0.3	4.0[5]	± 2.0
	nonimmunized	7	4.7	±2.5	5.3[3]	±3.4	20.5	±13.6	0.2	±0.1	33.0	±10.2
30	immunized	3	0.4	±0.1	0.3	±0.0	47.6	± 8.1	0.4	±0.1	1.0[4]	± 0.0
	nonimmunized	3	3.5[3]	±0.4	0.6	±0.9	34.0	± 3.1	0.4	±0.0	8.0	± 2.3
45	immunized	3	1.4[4]	±1.4	0.7	±0.4	39.2	± 2.9	0.4	±0.2	2.0[4]	± 1.7
	nonimmunized	3	2.7[3]	±0.4	3.5[3]	±1.7	28.9	± 4.0	0.2	±0.0	16.0	± 2.4
60	immunized	10	1.3[3,4]	±0.9	0.7[3,4]	±0.4	37.5	±21.8	0.3	±0.2	3.0[6]	± 1.6
	nonimmunized	15	4.8[3]	±4.0	6.0[3]	±4.7	31.9	±13.5	0.4	±0.0	12.0	±23.0

[1] Values are given as mean ± standard deviation of per cent injected dose.
[2] Labelled 1×10^9 Type II pneumococci were injected intravenously into adult immunized or nonimmunized rabbits. The animals were sacrificed at the indicated intervals and weighed aliquots of the spleen and liver were assayed for radioactivity.
[3] $p = 0.05$ for difference between this and corresponding measurement of liver.
[4] $p = < 0.01$ for difference between this and corresponding measurement in nonimmune group.
[5] $p = 0.02$ for difference between this and corresponding measurement in nonimmune group.
[6] $p = 0.05$ for difference between this and corresponding measurement in nonimmune group.
Significance determined by the *t*-test.

Table II. Uptake of I^{125}-labelled pneumococci[1]

Experimental procedure	Number in group	Spleen				Liver				Per gram spleen	
		Per organ		Per gram		Per organ		Per gram		Per gram liver	
Immunized[2]: T.I; challenged: I^{125} T.I	4	0.5	±0.3	0.3	± 0.3	25.3	± 7.5	0.3	±0.1	1	± 0.6
Immunized[2]: T.II; challenged: I^{125} T.I	7	18.8	±9.2	17.2	±10.0	32.5	±21.7	0.3	±0.2	61	±28.5
Given[3]: S.II; challenged: I^{125} T.II	4	3.5	±2.1	2.8	± 0.9	30.0	± 2.3	0.3	±0.1	10.0	± 3.5
Nonimmunized; challenged[3]: T.II	4	4.0	±0.6	8.0	± 2.2	34.7	±13.1	0.4	±0.3	25.0	±10.7
Given[4]: T.II antiserum; challenged: I^{125} T.II	4	0.6	±0.1	0.4	± 0.1	30.1	±10.8	0.3	±0.1	2	± 1.0
Given[4]: T.III antiserum; challenged: I^{125} T.II	4	12.6	±6.3	8.9	± 5.5	47.8	± 9.3	0.6	±0.1	15	±10.2

[1] Values are given as mean ± standard deviation of per cent injected dose.
[2] Rabbits immunized as indicated were given 1×10^9 I^{125} Type I (T.I) or Type II (T.II) pneumococci intravenously and the organ distribution in liver and spleen was assayed 60 minutes after challenge.
[3] Rabbits were given Type II pneumococcal polysaccharide (S.II) 3 mg in 3 divided doses intravenously, then challenged 7 days later with labelled 1×10^9 Type II (T.II) pneumococci and the organ distribution assayed 60 minutes later.
[4] Rabbits were given 2,000 I.U. Type II or 4,000 I.U. Type III pneumococcal antiserum intravenously in 1 ml, and challenged one hour later with labelled 1×10^9 Type II (T.II) pneumococci; organ distribution was assayed 60 minutes later.

examined. One week following three weekly intravenous injections of 1 mg of pneumococcal polysaccharide Type II, groups of rabbits were challenged with type specific I^{125} pneumococci, and clearance compared with that of nonimmunized controls. The results are summarized in table II. No statistically significant differences were found between the mean uptakes in control rabbits and in those receiving the polysaccharide injections; however, the mean uptake per gram of spleen was consistently lower in the experimental groups than in their controls or in other control groups receiving comparable injections of Type II pneumococci. The significance of this result is not clear at present, and is being investigated further. These results are, however, compatible with the type specificity of the patterns of uptake observed in immune rabbits.

To further confirm type specificity and to determine whether the observed effects were antibody-mediated, rabbits were given 2000 I.U. of antipneumococcic serum Type II or 4000 I.U. of antipneumococcic serum Type III and challenged with I^{125}-labelled Type II pneumococci; one hour later spleen and liver uptake were determined. The results are summarized in table II. The animals which had received the anti-pneumococcic Type III serum had the high splenic uptake response characteristic of nonimmune rabbits, whereas those which had received specific antisera cleared pneumococci like actively immunized animals.

Serum was obtained from the rabbits, just prior to their being challenged with labelled bacteria, and was examined for specific antibody activity to the capsular polysaccharide, using the Quellung reaction. No antibody was detected in any of the experimental groups except in those animals which had been passively immunized.

Discussion

The studies reported here confirm the long established observation that pneumococci are cleared from the circulation very rapidly in the immune as contrasted to the normal rabbit. The patterns of uptake by the liver and spleen were compared both in terms of activity per unit of weight, and as whole organs at various intervals during the initial clearance of labelled organisms. Marked differences in the patterns were found in actively or passively immunized, specifically paralyzed, and untreated groups of rabbits.

Per unit of wet weight, the spleen was the more active of the two organs in both the immune and nonimmune groups. In various experiments the immune spleen accumulated one to ten times the concentration of radioactivity of the liver on this basis; however, the spleen was most strikingly active in the nonimmune groups, in which case the concentration of radioactivity was up to 60 times that found in the liver, and the average value was in the range of 18. When considered in terms of proportion of the injected dose taken up into the whole organ at the various intervals, the liver dominated uptake in both normal and immune groups. In the nonimmune group, the liver contained two to ten times the amount of radioactivity of the spleen at all intervals; in the immune group, differentials of up to 100 times this value were found. The immunological type specificity of antibody mediated clearance patterns was confirmed.

Thus it was found that the spleen has the higher capacity for clearance of pneumococci regardless of the immunological status of the animal, but that this role is most significant in the nonimmune animal. The liver, in contrast, with a lower capacity for clearance per unit of weight, accumulates a far greater proportion of cleared organisms, but depends heavily upon the presence of type specific antibody to engage its clearance capacity maximally.

The importance of specific antibody in the greater overall uptake of labelled pneumococci by the liver seems clear. Although information is not available for the rabbit, studies of the relative cellular content of the human and rat liver have shown that 14–15 % wet weight in humans [10] and 30–38 % wet weight in the rat [6], consist of macrophages or littoral cells. These fixed macrophages are in direct contact with the portal circulation. Thus when circulating bacteria or other antigenic materials are specifically opsonized by antibody, this large reserve of phagocytic capacity can function with great efficiency in the rapid clearance of the circulation. In the experiments described here this opsonization of antigen occurred prior to 15 minutes, the first sampling time. When the circulation is cleared of organisms so rapidly, an insufficient period of bacteremia probably elapses for the bulk of organisms to accumulate in the spleen by any mechanism other than that which occurs in the liver—simple opsonin dependent phagocytosis. When no specific antibody is present to permit efficient liver uptake, clearance is thus delayed, permitting more rate-limited and specialized uptake mechanisms in the spleen to function in presence of continued bacteremia.

Such a mechanism appears to exist in the white pulp marginal sinuses of the spleen (the follicular web of the spleen) where antigen is trapped as described by WHITE [19] and by NOSSAL *et al.* [13]. Intravenously injected antigens appear to concentrate initially in this region after evanescent appearance in the red pulp. Direct evidence that I^{125}-labelled pneumococci are trapped in significant quantity in the cortical area of the spleen during the first hour is lacking here, and this is being investigated currently.

The possibility that the preferential acquisition of particulate antigens in the absence of antibody may favor primary immunological function of the spleen is suggested by the studies in rats and in humans by ROWLEY [16, 17]. He showed that a small amount of particulate antigen given intravenously stimulated little or no antibody response in splenectomized individuals as compared to controls with intact spleens.

Indirect evidence of a rapid specific immune response by the rabbit spleen is available. In the newborn rabbit, evidence for specific γM antibody synthesis to *S. typhimurium* is seen in the spleen as early as 16 hours after initial contact with antigen by the intraperiotoneal route [1]. Preliminary studies in this laboratory indicate that the administration of a single intravenous injection of killed organism to rabbits, as little as 8 hours, before challenge with living virulent pneumococci decreased the time necessary for clearance of these organisms and prevented a lethal outcome [7]. This protection was type specific and apparently localized to the spleen, for no antibody was detectable in the circulation and the protective effect failed to occur in splenectomized animals. Such a pulse dose failed to alter the uptake pattern of I^{125}-labelled pneumococci as might be expected. Similar studies have shown that splenectomy increases susceptibility of mice to infection following intravenous injections of pneumococci [18].

The unique capacity of the spleen for clearance of bacteremia, in the absence of specific antibody, may have its significance in the initiation of a rapid local specific immune response and insuring more effective control of subsequent phases of bacteremia. When immunological experience is limited, as in the young individual, the absence of such a protective mechanism, as after splenectomy or when the spleen is functionally impaired, might be a major factor in determining the outcome of bacteremia associated with otitis or pneumonia.

Summary

The clearance of labelled pneumococci as a function of the liver and spleen of mature rabbits, in the presence and absence of specific antibody, were studied. The results indicated that the spleen has the greater capacity for clearance regardless of immunological status of the animal, but that this role was most significant in the nonimmune animal. Mechanisms explaining these phenomena and their possible biological significance are discussed.

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