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The Primary Immune Response in Mice

III. Retention of Sheep Red Blood Cell Immunogens by the Spleen and Liver

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The antibody-inducing activities of foreign red blood cell immunogens sequestered in the spleens and livers of mice injected with sheep erythrocytes were evaluated during the early periods of the immune response. Estimates of immunogenicity, obtained from the magnitudes of anti-sheep red blood cell hemolysin responses evoked in sensitized recipient mice by subcellular tissue fractions prepared from these phagocytic organs, showed that the liver and spleen differ greatly in their handling of this particulate antigen. The liver, functioning primarily as a scavenger organ, destroys completely the immunogenicity of the heterologous erythrocytes in 12 hr. In contrast, the spleen handles foreign erythrocyte immunogens in at least two different ways: approximately 90% of the initially sequestered activity is rapidly destroyed by the spleen in 6 hr, but the remaining activity, associated chiefly with a tissue fraction possessing the sedimentation properties of "light mitochondria," is retained at a significant level for 3 days, and then progressively decreases to a low level until the 7th day. A correlation of the observed changes in the properties of the immunogenic tissue fraction with known cellular events in the spleen stimulated by antigens indicates that the retention of the degradable erythrocyte immunogen is essential for stimulating and maintaining immune reactions in this antibody-producing organ.

How information for three-dimensional structural specificity is transferred from the antigen to a class of immunocompetent cells is not known. One approach to an understanding of this central problem in immunology is to study the immunogenic form of a fundamentally degradable antigen retained by lymphoid tissues during the course of the immune response. Tissue-retained antigens have previously been shown to be important in the production and maintenance of immune phenomena (6, 31, 45, 49, 55, 64) and for the complete differentiation of immunocompetent cells (25, 29, 56).

The persistence of immunologically active material in the animal body varies with both the antigen and the species of animal used in experiments, as clearly shown in the reviews of Humphrey (26), Campbell and Garvey (7), and Sulitzeanu (57). For example, diphtheria toxin remains immunogenic in rabbit lymphoid tissue for 3 weeks (53), and bovine gamma globulin remains immunogenic in rabbit livers for 14 days (35); furthermore, pneumococcus polysaccharide antigen persists in various tissues of "paralyzed" mice in an immunogenic form for very long periods (14). Recently, studies of the ability of retained antigens to initiate an immune response in nonsensitized syngeneic mouse lymphoid cells transferred to primary antigen-injected recipients have demonstrated that sheep red cell immunogens persist for 14 days, whereas lipopolysaccharide antigens remain immunogenic for at least 45 days (6). Likewise, stimulatory material was still detected in host mice 10 weeks after their injection with 100 mg of bovine serum albumin by the immune responses of transferred sensitized spleen cells (41).

The present experiments continue our earlier studies of the primary immune response in the mouse (17, 36) and bring out new information about the immunological behavior of tissue-retained antigens during the early phases of the immune response. This has been accomplished by comparing the immunogenic capabilities of injected heterologous erythrocytes sequestered by the spleen, an avid producer of antibodies, with the antibody-stimulating activity of red blood cells taken up by the liver, an organ normally devoid of this function. Estimates of the immunogenicity of retained foreign erythrocyte antigens were obtained from the magnitudes of the anti-sheep red cell hemolysin responses

evoked in presensitized recipient mice by subcellular fractions prepared from these phagocytic organs. In passing, it should be noted that others have employed tissue fractionation techniques to study the immunological history of bacteriophages retained by spleens and livers of guinea pigs (63) and of sheep erythrocytes sequestered by the spleens of rats (50). Preliminary communications of our studies have been previously reported (16; R. E. Franzl and E. Leckband, Fed. Proc. **23**:344, 1964).

MATERIALS AND METHODS

Mice. Female mice of the Rockefeller Swiss Nelson-Collins strain, 4 to 6 weeks old and weighing 20 to 22 g, were employed in all experiments. The animals were housed at 22 ± 1 C, fed *ad libitum*, and given drinking water containing 1 mg of oxytetracycline (soluble powder, Pfizer Co., Inc., New York, N.Y.) per ml.

Antigen. Sheep red blood cells (RBC; Cappel Laboratories, West Chester, Pa.), washed three times by centrifugation with sterile saline, were employed as antigen in these studies. The cell suspensions were adjusted to the desired concentration by standardization in terms of hemoglobin. With this method, lysis of a 1% sheep RBC suspension (2×10^8 cells per ml) with 14 volumes of 0.1% Na_2CO_3 develops an optical density of 0.135 at 541 nm in a Beckman DU spectrophotometer.

Treatment of animals. Donor mice were injected intravenously with 2×10^8 sheep RBC, and, at specified time intervals thereafter, their tissues were removed for subcellular fractionation as described below. In general, recipient mice were presensitized with a single intravenous injection of 2×10^6 sheep RBC 5 to 6 weeks before being used in the immunogenicity assay. In some instances, however, normal untreated mice were used as recipient animals.

Tissue fractionation. The subcellular fractions of liver and spleen were prepared essentially by the differential centrifugation method of de Duve and coworkers (11), with the use of a medium containing 0.25 M sucrose, 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 0.005 M $MgCl_2$, and 0.03 M KCl buffered at *p*H 7.6. In typical experiments, separate pools of approximately 22 livers and 40 spleens were obtained from donor mice previously injected with sheep RBC. Each tissue was homogenized with an amount of medium calculated to give a 25% (w/v) tissue extract by one up and down stroke of the motor-driven plunger of a Potter-Elvehjem type homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.); the tissue extract was centrifuged at $1,000 \times g$ for 10 min in a centrifuge with swing-out cups (International Centrifuge Co., Boston, Mass.), and the supernatant fluid was saved. These operations were repeated twice with the pellet while employing a force of $600 \times g$ for the 10-min centrifugations. The final sediment, resuspended and passed through flannelette, was called the nuclear fraction (N). The pooled supernatant fluids, centrifuged at $3,000 \times g$ for 10 min in an angle-head rotor (ultracentrifuge model L, Beckman Instruments Inc., Spinco Division, Palo Alto, Calif.), yielded a pellet which, after being washed twice by resuspension and sedimentation, was designated as mitochondria (M). By centrifuging the combined supernatant fluid and the fluids of the washings at $25,000 \times g$ for 10 min, a third pellet of "light mitochondria" (L) was obtained. The supernatant fluid yielded the particulate microsome fraction (P) after 50 min of centrifugation at $60,000 \times g$. The unsedimentable material constituted the cell supernatant fluid (S). All procedures were done in the cold (0 to 4 C). The pellets were resuspended directly in their centrifuge tubes with a loosely fitted motor-driven smooth Teflon pestle. The final suspension of subcellular particulates was made in 0.05 M Tris-hydrochloride buffer (*p*H 7.4) containing 0.005 M $MgCl_2$ and 0.03 M KCl.

Assay of immunogenicity. The immunogenic activity of injected sheep RBC retained by the liver and spleen was assayed by testing the capabilities of subcellular fractions prepared from these organs of donor mice to elicit anti-sheep RBC hemolysin responses in sensitized recipients. At the time of assay, each tissue fraction, isolated as described above, was carefully injected intravenously into 9 or 10 anesthetized recipient mice which 3 min previously had been given 500 USP units of heparin (Bio Heparin, Ries Biologicals, Los Angeles, Calif.) to avoid intravascular clotting (22). The recipients' sera contained no detectable amounts of hemolytic antibody at this time. Sera pooled from the brachial bleedings of three to four recipient mice were collected on the 4th, 6th, and 8th days after injection, stored at −20 C, and titered for their content of anti-sheep RBC hemolysin as described below. In some experiments, sera were collected over a period of 14 days. By matching the kinetics of the serum hemolysin responses elicited by donated tissue fractions in sensitized recipients with those of the "standard" responses evoked by known amounts of sheep RBC in identically sensitized mice (see Fig. 1), an approximation of the quantity of foreign red cell immunogens associated with each cell fraction is obtained in terms of an equivalent number of sheep erythrocytes.

Immune responses were also followed by the number of "direct" (19*S*) hemolysin plaque-forming cells (PFC) in the spleens of recipient mice on successive days after immunization, as determined by the Jerne, Nordin, and Henry agar plaque technique (30).

Antibody determination. Quantitative titrations of anti-sheep RBC hemolysins (60) were performed on antisera decomplemented by heating at 56 C for 0.5 hr. The results appear as the logarithm of the number of 50% anti-sheep RBC hemolysin units per milliliter of undiluted mouse serum ($\log_{10}K$). A control titration of a standard hemolysin preparation accompanied each set of determinations.

Nitrogen determination. A modified nesslerization procedure (5, 42) was employed to quantitate 5 to 80 μg of nitrogen.

RESULTS

Hemolysin responses of sensitized mice to various doses of sheep RBC. To establish the degree of responsiveness to sheep erythrocyte

immunogens in the sensitized mice regularly employed as recipients in the immunogenicity assay (see Material and Methods), a study was made of the secondary serum hemolysin responses evoked in these animals by single intravenous injections of 10-fold dose increments of antigen ranging from 2×10^4 to 2×10^8 sheep cells. An examination of the resultant responses illustrated in Fig. 1, with regard to antibody production kinetics, maximal titers attained, and the time of the first appearance of antibody, reveals that each stimulatory dose of foreign red cells, within the 1,000-fold range of antigen dosage, evoked its own distinctive production of serum hemolysins. Thus, 2×10^4 sheep RBC elicited a minimal response with a latent period of 5 days (curve a); the larger dose of 2×10^5 cells induced a considerable production of antibody commencing 4 days after antigen administration (curve b). In contrast, 2×10^6 foreign erythrocytes stimulated a rapid response with hemolysins already in evidence on day 3 (curve c), and 2×10^7 blood cells initiated a maximal production of anti-sheep RBC hemolysins in sensitized mice (curve d). As previously discussed (Materials and Methods), these anti-sheep RBC hemolysin responses are used as "standards" to approximate the quantity of the sheep red cell immunogens retained by livers and spleens of antigen-injected mice in the experiments described below.

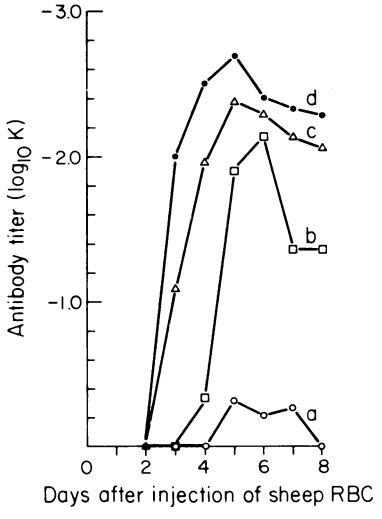

FIG. 1. *Effect of the dosage of injected antigen on the production of secondary anti-sheep RBC hemolysins in mice. The figure shows the serum hemolysin responses elicited by single intravenous injections of* 2×10^4 *(a, ○),* 2×10^5 *(b, □),* 2×10^6 *(c, △), and* 2×10^7 *(d, ●) sheep RBC in mice primed 5 weeks previously with* 2×10^6 *sheep RBC. The antibody titers* (log_{10}K) *are expressed as the logarithm of the number of 50% anti-sheep RBC hemolysin units per milliliter of undiluted serum. Each point represents the antibody titer of sera pooled from three animals.*

Persistence of sheep red cell immunogens in the liver after the injection of sheep RBC. To investigate the retention of heterologous RBC immunogens by the liver, the levels of sheep red cell immunogenicity were determined in liver subcellular fractions "N," "M," "L," and "P" (see Materials and Methods), prepared 1, 3, 6, and 12 hr after the intravenous injection of donor mice with 2×10^8 sheep RBC. The ability of the donated tissue fractions to stimulate anti-sheep RBC hemolysin responses in previously sensitized recipient mice was utilized to test for the presence of foreign RBC immunogens in the liver preparations. From the magnitude of the responses depicted in the graph on the left in Fig. 2, it is evident that all tissue preparations made from the donor's liver 1 hr after antigen were highly immunogenic, and that "L" and "P" fractions were possibly more active than either of the two remaining subcellular particles. The latter is definitely true for samples obtained 3 to 6 hr after antigen. This fact is supported by the data in the second and third diagrams in Fig. 2, as shown by the contrast of the sustained high titers elicited by the lighter cytoplasmic fractions with the low responses evoked by similar injections of "N" and "M" cell particulates. However, most cell fractions obtained 12 hr after antigen injection were totally devoid of sheep cell immunogenicity (note the absence of significant responses in the graph on the right), with the exception of "L" preparations exhibiting a trace of biological activity. Thus, it is evident that most of the antibody-inducing activity of the foreign RBC initially sequestered by the liver is destroyed after a 12-hr residence in this phagocytic organ. Subsequently, all subcellular fractions prepared 1 to 7 days after antigen and presented in doses of 1.0 to 1.2 mg of nitrogen per mouse were found to be totally incapable of stimulating the production of anti-sheep RBC hemolysins in sensitized recipient mice.

The evaluations of the immunogenicities of each donated subcellular preparation in terms of equivalent numbers of sheep erythrocytes (see Materials and Methods), listed in the third column of Table 1, establish with certainty that liver "L" and "P" fractions contain large amounts of red cell immunogens at 1 hr, less at 3 and 6 hr, and virtually none at 12 hr after the injection of 2×10^8 sheep RBC. In addition, the liver's rapid destruction of sheep cell immunogenic activity is further substantiated by the parallel decline of the calculated "specific" immunogenicities of the cell

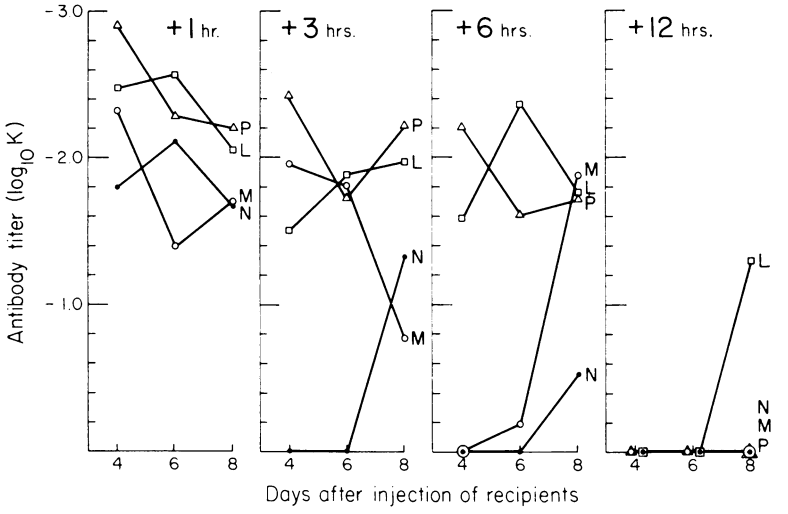

FIG. 2. *Anti-sheep RBC hemolysin responses induced by liver cell fractions obtained from donor mice over a 12-hr period after antigen administration. Cell fractions, nuclei (N, ●), mitochondria (M, ○), "light mitochondria" (L, □), and microsomes (P, △) were prepared from the livers of donor mice +1, +3, +6, and +12 hr after intravenous injections of 2 × 10^8 sheep RBC. The fractions were intravenously injected into sensitized recipient mice in the following doses (milligrams of nitrogen per mouse): in the +1-hr experiment—N (0.45), M (0.58), L (0.97), and P (0.79); in the +3-hr experiment—N (0.58), M (0.41), L (0.78), and P (0.74); in the +6-hr experiment—N (0.31), M (0.27), L (0.63), and P (0.49); and in the +12-hr experiment—N (0.32), M (0.32), L (0.37), and P (0.36). The antibody titer (log_{10} K) of recipient mice is expressed as the logarithm of the number of 50% anti-sheep RBC hemolysin units per milliliter of undiluted serum.*

particulates over the 12-hr period (fourth column, Table 1).

Persistence of sheep red cell immunogens in the spleen after the injection of sheep RBC. To determine the sheep RBC immunogenicity retained in the spleens of antigen-treated donor animals, subcellular fractions, prepared from this organ 1 to 12 hr after the administration of sheep erythrocytes were given to sensitized recipients in doses approximating one to three spleens per mouse. The results of these experiments, illustrated in Fig. 3, show that "1-hr" spleen fractions (first graph of Fig. 3) elicited greater anti-sheep RBC hemolysin responses in the test animals than did the "3-, 6-, and 12-hr" fractions, the responses of which are represented by the corresponding plots in Fig. 3. The findings clearly demonstrate the large measure of immunogenic capability associated with spleen cell particulates obtained directly after the injection of antigen, and the somewhat diminished activities of analogous preparations isolated later during the 3- to 12-hr period. However, it must be emphasized that all cytoplasmic particulates isolated from the spleen throughout the 12-hr period after antigen consistently evoked sizable antibody titers on all 3 test days and can, therefore, be considered to contain appreciable amounts of foreign immunogens. This is in contrast to the previously presented data demonstrating a rapid destruction of sheep RBC immunogenicity associated with liver subcellular fractions during the same experimental period (see Fig. 2 and Table 1).

To investigate the eventual fate of foreign RBC immunogens in the spleen, a study was made of the hemolysin-inducing capability of splenic fractions obtained 1, 2, 3, and 4 days after the injection of the optimal dose of sheep RBC. From the data presented in the first three graphs of Fig. 4, it is evident that fractions obtained from donor animals 1 to 3 days after antigen injection possessed considerable immunogenic activity. This was especially true of "L" fractions isolated within this 3-day interval. They consistently elicited high hemolysin titers in sensitized mice on the 4th day after their administration, whereas, as shown in these graphs, similar doses of "M," "N," and "P" fractions evoked considerably lower responses. In contrast, "4-day" tissue preparations (right plot of Fig. 4) either were inactive ("N" and "M" fractions) or exhibited only token immunogenicity ("L" and "P" fractions). Further studies showed that spleen "L" fractions, isolated 7 days after antigen administration, were still very slightly immunogenic, whereas fractions obtained 30 days later, contained no demonstrable activity.

An evaluation of the sheep cell immunogenicity

TABLE 1. *Sheep red cell immunogenicity of liver cell fractions at various time intervals after antigen injection*

Time after injection (hr)[a]	Cell fraction[a]	Immunogenicity[b]	"Specific" immunogenicity[c]
+1	N	0.7×10^6	1.55×10^3
	M	0.7×10^6	1.21×10^3
	L	7.0×10^6	7.20×10^3
	P	20.0×10^6	25.6×10^3
+3	N	0.02×10^6	0.03×10^3
	M	0.7×10^6	1.71×10^3
	L	2.0×10^6	2.56×10^3
	P	2.0×10^6	2.70×10^3
+6	N	0	0
	M	0.07×10^6	0.26×10^3
	L	2.0×10^6	3.18×10^3
	P	2.0×10^6	4.08×10^3
+12	N	0	0
	M	0	0
	L	0.02×10^6	0.05×10^3
	P	0	0

[a] For explanation of symbols, see legend to Fig. 2.

[b] The immunogenicity of the injected dose of the liver cell fractions (Fig. 2) is expressed as the number of sheep RBC necessary to evoke a comparable secondary hemolysin response in sensitized recipient mice (see Materials and Methods).

[c] "Specific" immunogenicity is the immunogenicity per microgram of nitrogen of the cell fraction.

found in spleen cell fractions obtained 1 to 12 hr and 1 to 4 days after antigen injection in terms of an equivalent number of sheep RBC is presented in Tables 2 and 3, respectively. The results in Table 2 demonstrate the loss of a major portion of the spleen's early (+1 hr) sheep cell immunogenicity after 3 hr and the retention of a significant antibody-stimulating activity throughout the remainder of the 12-hr experimental period. This activity was shown to persist in the spleen for an additional 3 days (Table 3) and to associate principally with the "light mitochondrial" ("L") fraction.

The immunogenic activity of comparable doses of spleen "L" fractions, isolated from donor animals 1, 2, 3, and 4 days after treatment with sheep RBC, was also studied by employing the "direct" hemolysin plaque technique in agar (30) to enumerate (19*S*) antibody-producing cells in the spleens of the sensitized recipient mice. The results of these investigations are plotted in the right diagram of Fig. 5, each point representing the average number of PFC per 10^6 spleen cells in a pool of three to four recipient spleens. As can be seen, fractions isolated 1 day after antigen administration gave the greatest response (curve 1), and those obtained at later time intervals evoked successively decreasing responses (curves 2, 3, and 4). These prompt and brisk reactions resemble closely the secondary hemolysin response (Fig. 5, left plot) obtained by challenging similarly sensitized mice with sheep erythrocytes.

Further properties of spleen "light mitochondrial" fractions from antigen-injected mice. To characterize the nature of the biologically active sheep RBC immunogens associated with splenic "L" subcellular particles, a study was made of the effects of several experimental procedures on the capabilities of these preparations, obtained 1 and 3 days after antigen injection, to stimulate the production of anti-sheep hemolysins in sensitized mice. Table 4 compares the immunogenic activities of treated and untreated tissue fractions with those of sheep erythrocytes. As can be seen, both the tissue preparations and the blood cells retained their initial sheep cell immunogenicity for considerable time periods when maintained at 4 C in the buffer usually employed in these investigations. In addition, dialysis of similar preparations for 16 hr at 4 C against the same buffer (0.05 M Tris buffer) devoid of Mg and K ions had no effect on their activities. In contrast, incubation at 37 C (see second line of Table 4) greatly impaired the biological activities of both spleen fractions, although the procedure still had no effect upon the antibody-stimulating activity of lysed sheep RBC, even when spleen "L" fractions were included in the incubation mixtures.

The action of specific antibodies on the immunogenic activity of tissue-retained antigens was investigated by reacting each of the spleen "L" preparations with high-titer mouse anti-sheep RBC hemolysin serum ($\log_{10} K$, -3.5). The results shown on line 3 of Table 4 indicate that antigenic determinants of sheep immunogens associated with the "1-day" subcellular fractions reacted with antibody and formed immunologically inactive material, whereas those present in the later "3-day" fractions appeared to be largely protected from the inactivation by specific antibody. These preliminary observations suggest that heterologous RBC immunogens, present in the spleen 1 day after antigen injection, differ appreciably, either in chemical structure or in their association with cellular components, from those found on the 3rd day. Since the reaction of antibody with sheep erythrocytes did not influence the antibody-stimulating activity of this

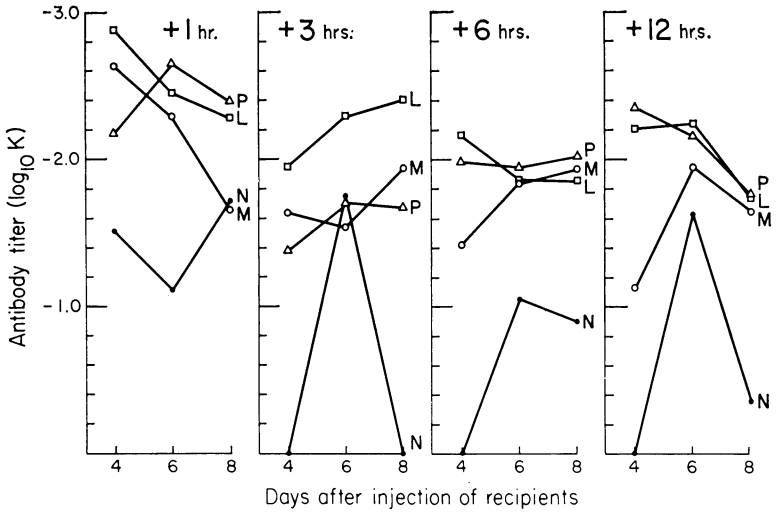

FIG. 3. *Anti-sheep RBC hemolysin responses induced by spleen cell fractions obtained from donor mice over a 12-hr period after intravenous injections of sheep cells. The doses (milligrams of nitrogen per mouse) of each fraction administered to sensitized recipient mice were: in the +1-hr experiment—N (0.66), M (0.35), L (0.30), and P (0.39); in the +3-hr experiment—N (0.53), M (0.37), L (0.17), and P (0.35); in the +6-hr experiment—N (0.35), M (0.19), L (0.23), and P (0.26); and in the +12-hr experiment—N (0.41), M (0.23), L (0.23), and P (0.32). For explanation of symbols, see legend to Fig. 2.*

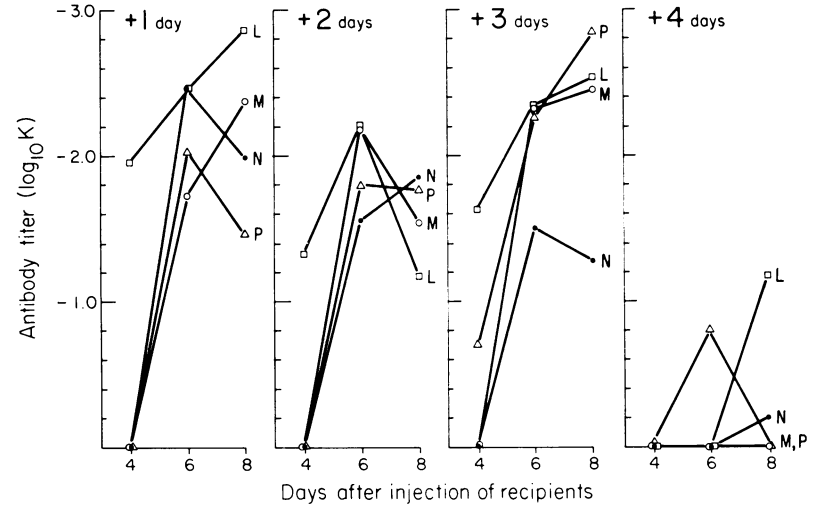

FIG. 4. *Anti-sheep RBC hemolysin responses induced by spleen cell fractions obtained from donor mice over a 4-day period after antigen injection. The doses (milligrams of nitrogen per mouse) of each fraction given to sensitized recipient mice were: in the +1-day experiment—N (0.91), M (0.40), L (0.33), and P (0.61); in the +2-day experiment—N (0.81), M (0.46), L (0.49), and P (0.43); in the +3-day experiment—N (0.41), M (0.39), L (0.32), and P (0.44); and in the +4-day experiment—N (0.95), M (0.11), L (0.48), and P (0.46). For explanation of symbols, see legend to Fig. 2.*

particulate antigen, the mechanism by which the immunogenicity of "L" tissue fractions is inactivated by serum antibody is probably different and is not presently understood.

In an attempt to remove sheep RBC immunogens from tissue components, spleen "L" fractions were treated with a surface-acting agent, sodium deoxycholate. As Table 4 shows, the immunogenic activity of lysed sheep RBC and spleen "1-day" fractions was not affected, as all activity

TABLE 2. *Sheep red cell immunogenicity of spleen cell fractions at various times during a 12-hr period after antigen injection*

Time after injection (hr)[a]	Cell fraction[a]	Immunogenicity[b]	"Specific" immunogenicity[c]
+1	N	0.2×10^6	0.3×10^3
	M	7.0×10^6	20.0×10^3
	L	20.0×10^6	66.6×10^3
	P	7.0×10^6	17.9×10^3
+3	N	0.07×10^6	0.13×10^3
	M	0.7×10^6	1.89×10^3
	L	7.0×10^6	41.2×10^3
	P	0.7×10^6	2.0×10^3
+6	N	0.02×10^6	0.06×10^3
	M	0.7×10^6	3.68×10^3
	L	2.0×10^6	8.70×10^3
	P	2.0×10^6	7.70×10^3
+12	N	0.07×10^6	0.17×10^3
	M	0.7×10^6	3.04×10^3
	L	2.0×10^6	8.70×10^3
	P	2.0×10^6	6.25×10^3

[a] For explanation of symbols, see legend to Fig. 2.

[b] The immunogenicity of the injected dose of the spleen cell fractions (Fig. 3) is expressed as the number of sheep RBC necessary to evoke a comparable secondary hemolysin response in sensitized recipient mice (see Materials and Methods).

[c] "Specific" immunogenicity is the immunogenicity per microgram of nitrogen of the cell fraction.

TABLE 3. *Sheep red cell immunogenicity of spleen cell fractions at various times during a 4-day period after antigen injection*

Time after injection (days)	Cell fraction[a]	Immunogenicity[b]	"Specific" immunogenicity[c]
+1	N	0.1×10^6	0.02×10^3
	M	0.1×10^6	0.25×10^3
	L	10.0×10^6	30.0×10^3
	P	0.02×10^6	0.03×10^3
+2	N	0	0
	M	0	0
	L	2.0×10^6	4.1×10^3
	P	0.02×10^6	0.05×10^3
+3	N	0.02×10^6	0.05×10^3
	M	0.1×10^6	0.26×10^3
	L	10.0×10^6	34.0×10^3
	P	1.0×10^6	1.7×10^3
+4	N	0	0
	M	0	0
	L	0.02×10^6	0.04×10^3
	P	0.02×10^6	0.05×10^3

[a] For explanation of symbols, see legend to Fig. 2.

[b] The immunogenicity of the injected dose of the spleen cell fractions (Fig. 4) is expressed as the number of sheep RBC necessary to evoke a comparable secondary hemolysin response in sensitized recipient mice (see Materials and Methods).

[c] "Specific" immunogenicity is the immunogenicity per microgram of nitrogen of the cell fraction.

was retained in sedimentable fractions, whereas the activity of the "3-day" preparations was completely destroyed by this reagent. The marked differences in lability to the action of the bile salt reemphasizes the previously postulated differences in the nature of sheep cell immunogens in spleen fractions obtained 1 day after antigen and of those associated with preparations obtained 3 days after antigen injection.

Ribonucleic acid (RNA) preparations (fraction I, ratio $A_{260}/A_{230} = 1.94$) derived from immunogenic spleen "L" fractions obtained 1 and 3 days after antigen injection by mild extraction with buffered phenol (21) were incapable of stimulating secondary hemolysin responses in vivo. Moreover, two additional fractions obtained by this extraction procedure, e.g., the phenol-soluble materials (fraction II) and the "interphase proteins" (fraction III) were, after removing all traces of phenol, also found to be immunogenically inactive (see Table 4). These results indicate that the biological activity of the immunogens under study is destroyed by this technique. To preclude the possibility of a destruction of the administered RNA by ribonuclease in the serum of recipients, RNA preparations were first adsorbed onto normal mouse spleen cells in hypertonic sucrose (37) prior to injection. Such RNA-bound cells still failed to stimulate an active production of anti-sheep RBC hemolysin in sensitized mice. Since immunogenic RNA could not be extracted from highly active spleen "L" fractions, and since the antibody-inducing capabilities of these tissue fractions were not altered by the action of ribonuclease in vitro, it may be postulated that RNA is not essential for the expression of sheep RBC immunogenicity associated with spleen "L" fractions of antigen-injected mice.

Primary immunogenic activity of spleen and liver "L" fractions. The activity of sheep cell immunogens present in subcellular tissue frac-

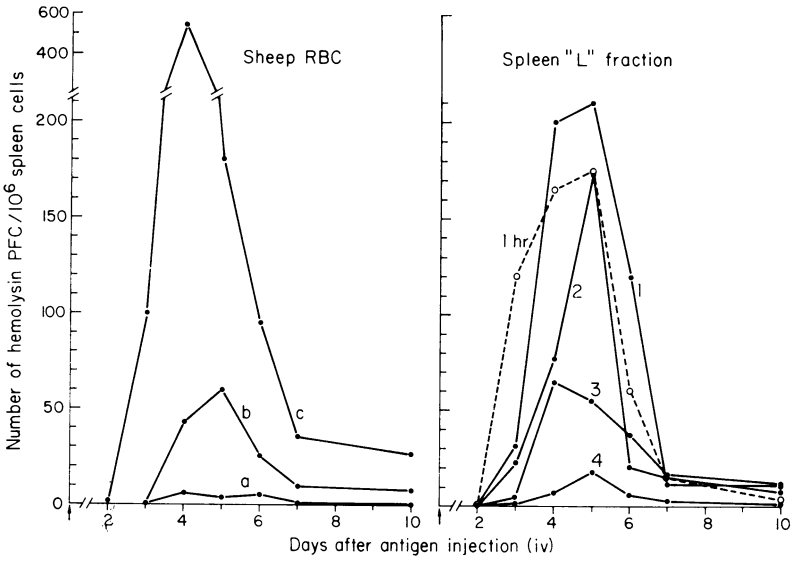

FIG. 5. *Production of splenic hemolysin plaque-forming cells (PFC) in sensitized recipient mice stimulated by different doses of sheep RBC (left figure) and by splenic "L" fractions isolated from donor animals at different times after giving 2 × 10⁸ sheep RBC (right figure). Left figure: Responses to the intravenous injection of 2 × 10⁴ (curve a), 2 × 10⁵ (curve b), and 2 × 10⁶ (curve c) sheep RBC. Right figure: Responses to intravenously injected spleen "L" fractions (milligrams of nitrogen per mouse) obtained 1 hr (curve 1 hr, 0.18), 1 day (curve 1, 0.16), 2 days (curve 2, 0.16), 3 days (curve 3, 0.19), and 4 days (curve 4, 0.26) after sheep RBC.*

TABLE 4. *Effects of various treatments on the immunogenic activity of sheep RBC and spleen "light mitochondrial" ("L") fractions*

Treatment	Duration and temp of treatment	Immunogenicity of treated material		
		sRBC[a]	SL + 1[b]	SL + 3[c]
Tris buffer, 0.05 M, pH 7.4	1 hr or 3 days, 4 C	100[d]	100	100
Tris buffer, 0.05 M, pH 7.4	1 hr, 37 C	100	5–10	5–10
Anti-sRBC serum, 3.2% in saline	30 min, 37 C	100	5	50
Sodium deoxycholate, 0.5%	4 C	50	100	0
Phenol, 88%	4 C			
Fraction I (RNA)			0	0
Fraction II			0	0
Fraction III			0	0

[a] Sheep RBC.
[b] Spleen "L" fractions prepared 1 day after the injection of 2 × 10⁸ sheep RBC.
[c] Spleen "L" fractions prepared 3 days after the injection of 2 × 10⁸ sheep RBC.
[d] Percent immunogenicity of the untreated material.

tions of antigen-injected donor mice was further investigated by evaluating the ability of spleen and liver "L" preparations to evoke immune responses in *normal* recipient animals. The results of a typical experiment (Fig. 6) show that 0.7 mg

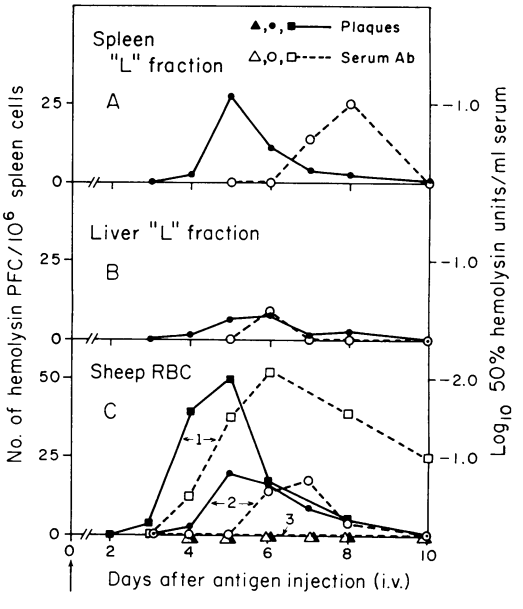

FIG. 6. *Responses in normal recipient mice stimulated by sheep RBC and by "L" tissue fractions (milligrams of nitrogen per mouse) isolated from donor animals 1 hr after the injection of 2 × 10⁸ sheep RBC. The primary production of splenic hemolysin plaque-forming cells (PFC; solid lines) and of serum hemolysins (dashed lines) evoked by the intravenous injection of (A) spleen "L" fraction (0.6), (B) liver "L" fraction (1.2), and (C) 2 × 10⁷ (curves 1, ■), 2 × 10⁶ (curves 2, ●), and 2 × 10⁵ (curves 3, ▲) sheep RBC.*

of nitrogen of an "L" spleen fraction obtained 1 hr after sheep RBC injection of donor mice stimulated anti-sheep hemolysin PFC and serum antibody responses in normal animals (see plot A) which are comparable to those evoked by 2×10^6 sheep RBC (plot C, curves 2). In contrast, a liver "L" fraction containing over twice this nitrogen content (1.7 mg of nitrogen) gave (see plot B) only one-third to one-fourth as great a reaction (compare plot B with plot A). These findings indicate that during this early period the spleen sequesters more immunologically active sheep erythrocytes per weight of tissue than the liver. However, since "L" fractions which were prepared from tissues later than 1 hr after injecting antigen, that is, 1 to 4 days later, did not stimulate immune responses in normal recipients, these "primary immunogens" appear to be short lived.

TABLE 5. *Retention of sheep red cell immunogenicity in liver and spleen after the injection of* 2×10^8 *sheep RBC*

Time after injection	Liver		Spleen		Recovery (%)[c]
	Immunogenicity (%)[a]	"Specific" immunogenicity[b]	Immunogenicity (%)[a]	"Specific" immunogenicity[b]	
1 Hr	59.5	40×10^2	10.5	93×10^2	70
3 Hr	8.5	6×10^2	3.0	27×10^2	11
6 Hr	10.5	8×10^2	1.5	15×10^2	12
12 Hr	0.04	0.5×10^2	1.0	13×10^2	1
1 Day	<0.04	$<0.5 \times 10^2$	1.0	13×10^2	1
2 Days	<0.04	$<0.5 \times 10^2$	0.2	3×10^2	0.2
3 Days	<0.04	$<0.5 \times 10^2$	1.0	13×10^2	1
4 Days	<0.04	$<0.5 \times 10^2$	0.05	0.6×10^2	0.05

[a] The total immunogenicity (equivalent number of sheep RBC) is expressed as the percentage of the injected dose of antigen.

[b] "Specific" immunogenicity is the immunogenicity per microgram of nitrogen.

[c] The sum of the immunogenicities of liver and spleen expressed as the percentage of the injected dose of antigen.

DISCUSSION

The immunological significance of antigens retained by tissues during the early phases of the immune response was evaluated by studying the sequential changes in the antibody-stimulating capacity of heterologous erythrocytes taken up by an immunocompetent organ, the spleen, and the changes of those sequestered by a largely phagocytic organ, the liver. To obtain precise data on these changes, subcellular tissue fractions, prepared at definite times after injecting mice with a single optimal dose of sheep RBC, were assayed for their ability to induce anti-sheep cell hemolysin responses in presensitized mice. The biological assay provided a direct evaluation of the functional activity of the sequestered antigen in relation to the immune response. It is known that the particulate antigen employed in these investigations is promptly removed from the circulation (22) and rapidly phagocytized by macrophages in the sinusoids of the spleen and by Kupffer cells of the liver. In addition, the uptake of foreign erythrocytes by the spleen stimulates the local production of specific antibodies in this organ (66), whereas sequestration of the immunogen by the liver appears to play no significant role in the resulting humoral response.

Data summarizing the fate of sheep RBC immunogens in the spleen are presented in the fourth column of Table 5. It is evident that 90% of the immunogenicity initially present in this organ was rapidly destroyed in 6 hr, and the remaining activity was retained at a significant level for 3 days and thereafter progressively decreased to a low level until the 7th day. These findings describing the fate of the immunogenic activity of sheep erythrocytes in the mouse spleen, as well as those reported by Hunter (27) and Nossal et al. (46) depicting the retention of ^{125}I-labeled flagella antigen by rat spleen, support the view that at least two mechanisms exist for handling particulate antigens in this antibody-producing organ. The first is ascribed to the activity of true phagocytic cells which localize antigens in their cytoplasmic organelles, phagolysosomes, and quickly degrade the ingested antigen and release the products of degradation (65). It explains the rapid inactivation of sheep RBC immunogenicity (Table 5) as well as the elimination, in 1 or 2 days, of most of the sequestered radioactivity labeled antigen (27, 46) from the macrophages of the marginal zone and the red pulp. Furthermore, the rapid inactivation of foreign red cell immunogenicity by phagocytes of the liver (column 2, Table 5) and by cells from the peritoneal cavity (18, 48), the destruction of the antibody-inducing activity of a soluble antigen by peritoneal macrophages (62), and the presence of antigens within phagolysosomes of cells in the marginal zone of the spleen (59), in the lymph node medulla (43), and in the peritoneal cavity (8, 13), all support the hypothesis that macrophages, not only in lymphoid tissues but in other parts of the organism as well, act primarily as scavengers when confronted with degradable antigens.

A key finding of this investigation is that a small but significant portion of a degradable antigen taken up by an immunocompetent organ is retained for an extended period in an immunogenic form. Thus, approximately 10% of the antigen sequestered by the spleen after a single dose of 2×10^8 sheep RBC is retained in an immunogenic form for at least 7 days (Table 5, Fig. 7A). These results are in essential agreement with those of Britton et al. (6) depicting the re-

tention of this immunogen by lethally irradiated mice for at least 14 days. The slight discrepancy in the times of immunogen retention may be explained, as previously expressed by these authors, by differences in sensitivity of the test system employed for immunogenic assay, and by the possibility of certain degrative processes occurring in vivo in a recipient animal which might destroy properties of the antigen molecule necessary for the induction of an immune response. It is now recognized that a number of antigens, which are retained by the spleen for prolonged periods, are localized in lymphoid follicles (27, 46, 58), primarily on the surface of infoldings of the plasma membrane of "dendritic reticular cells" which form a web within the germinal centers of lymphoid tissues (44, 59), in close association with lymphocytes and immunoblasts. In addition, it has also been proposed that extralysosomal sites for antigen sequestration also exist in phagocytic cells (8) with demonstrable immunogenic material retained on the plasma membrane (62), and of antigen label associated with the nuclear fraction of macrophages (32). Moreover, it is becoming evident that antigens also bind specifically to membranes of certain lymphocytes (1, 57). All such antigens or antigenic determinants, removed from the degradative action of the lysosomal enzymes of macrophages, would be in a position to exert their immunological specificities on immunocompetent cells, and consequently, as postulated by numerous investigators, to play a vital role in immune phenomena. Whether the prolonged retention of sheep RBC immunogens by the spleen represents an attachment of antigenic determinants to macrophages, dendritic reticular cells, or lymphocytes, or possibly to all three cell types, remains to be seen. The close association of retained sheep cell immunogens with a "nonadherent" cell population obtained from spleens of antigen-injected mice, reported recently by this laboratory (R. J. Bachvaroff and R. E. Franzl, Fed. Proc. **29**:825, 1970), suggests that lymphoid cells might play an important role in vivo in protecting these biologically active compounds from the degradative action of tissue phagocytes. Immunogens have also been found associated with the plasma membrane of peritoneal cells after the endocytosis of sheep RBC in vitro (10).

The significant level of immunogenicity retained by the spleen during the period from 1 to 3 days after the injection of sheep erythrocytes is associated primarily with a subcellular particle possessing the sedimentation characteristics of "light mitochondria" (Table 3). Because of the many types of cells present in lymphoid tissue, this fraction, which normally is rich in lysosomes and phagolysosomes (65), is very heterogeneous when obtained from the spleen (4). Moreover, it contains membranes, and, as shown by recent isopycnic centrifugation studies, a major part of the foreign red cell immunogens retained by the spleen is associated primarily with the membranes separated from "L" fractions of lymphoid cells obtained from this organ (R. Bachvaroff and R. E. Franzl, *in preparation*). Similar findings of Ada and Williams (3) demonstrated a firm binding of a serologically active material derived from the injected isotopically labeled bacterial flagella to cell residues, presumably membranes, associated with lysosomal-like particles of lymph nodes. It is therefore highly probable that the localization of sheep RBC immunogenicity in spleen "L" fractions represents retained immunogenic material attached to membranes which have accompanied the cytoplasmic fraction through the steps of its preparation. The continued study of the biochemical and immunological properties of these membranes is warranted, since these structures are intimately linked with the intercellular transmission of specific antigenic information for stimulating and maintaining immune reactions.

Immunogenic materials have also been demonstrated in subcellular fractions obtained from lymphoid tissues of antigen-injected animals of several species (50, 61, 63). Thus, spleen "L" fractions prepared from rats 1 day after the injection of sheep RBC were immunogenic in both normal and sensitized recipients, whereas those obtained 3 days after antigen, in the presence of actively produced antibody, did not stimulate responses in normal animals but retained their capacity to do so in previously immunized rats (50). These findings are at variance with the findings of the inability of 1- and 3-day mouse spleen "L" fractions, obtained both in the absence and in the presence of circulating antibody (see Fig. 7), to elicit responses in normal mice, and the demonstration of the ability of both fractions to induce the formation of secondary hemolysins. In addition, immunogenic "lysosomal" preparations have been derived from liver and spleen of rabbits up to 21 days after the injection of sheep RBC (61). The differences in immunogenic activity of sheep RBC retained by various animal species undoubtedly reflect the variety of reactivities, of stabilities, and, possibly, of "processing" pathways of the many antigenic determinants present on sheep erythrocytes. These differences suggest that, in a number of species, antibody production to this particulate antigen is elicited by dissimilar immunogenic determinants. Others (63), by comparing the antibody-inducing capa-

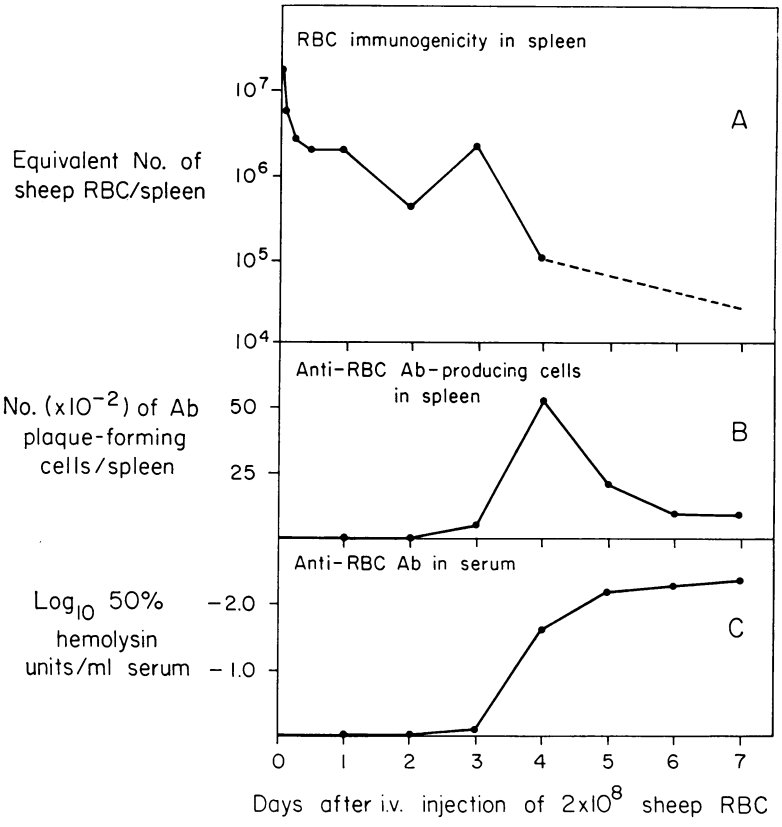

FIG. 7. *Retention of heterologous red cell immunogens by the spleen (A) and the accompanied production of splenic antibody-producing cells (B) and of serum hemolysins (C) after the injection of 2 × 10^8 sheep RBC. Each point represents an average of several experiments.*

bilities of a "large granular" cytoplasmic fraction obtained from spleens and livers of guinea pigs 1 and 48 hr after injecting bacteriophage, have suggested an immunological processing of retained immunogen.

In contrast to the prolonged retention of sheep red cell immunogens by the spleen, the liver of mice was found to destroy rapidly the bioloigical activity of the injected heterologous cell. Thus, 60% of the immunogenic dose sequestered by the liver's phagocytic cells 1 hr after antigen administration is reduced to a subthreshold level (below 0.04% of the given antigen) in 12 hr (see column 2, Table 5). In contrast, reports from other laboratories have shown that livers of guinea pigs still contained immunogenic material 48 hr after injection of bacteriophage (63), and those of rabbits harbored immunogenically active ^{35}S-labeled diazotized proteins for at least 24 hr (19). These longer periods of immunogen retention undoubtedly reflect differences in the handling of dissimilar antigens by this organ in the animal species employed. Furthermore, Garvey has shown (20) that, as a consequence of secondary antigenic challenge, a portion of the isotopically labeled antigen retained by the liver is released into the blood stream and subsequently becomes localized in lymphoid tissues. Since immunogens with sheep erythrocyte specificities were not detectable in livers of normal and of presensitized control mice directly before challenge, it follows that an analogous transfer of foreign red cell immunogenicity from liver to spleen did not occur within the detection limits of the assay system employed. In view of this, liver-retained antigens did not contribute to the reported amounts of foreign erythrocyte immunogens present in the spleens of mice injected with sheep red cells. Further, the spleen and liver differ markedly in their initial trapping efficiencies for heterologous erythrocyte immunogens. As shown by the data in columns 3 and 5 of Table 5, the spleen sequesters, per weight of tissue, approximately 10 times as much immunologically active antigen as the liver. These findings, as well as earlier reports of the unequal

retention of a variety of radioisotopically labeled particulate antigens by these organs in several animal species (12, 15, 27), and of the well-known differences in the abilities of these organs in mice to process serologically active streptococcal cell wall antigens (51) and to retain heterologous immune complexes (38), all point to the diverse handling of immunologically significant materials by the liver and the spleen.

Only a minute proportion of the originally administered immunogen plays a significant role in the immune response (16, 34). The retention, by an immunocompetent organ, the spleen, of approximately 1% or less of the dose of an easily degradable antigen in an immunogenic form during the early periods of an immune response (column 6, Table 5) tentatively suggests the possibility that this biologically active material plays a significant role in stimulating and maintaining the immune reactions in this organ. Thus, the properties, fate, and probable sites of localization of captured sheep RBC immunogens correlate well with the known cellular events in the spleen which lead to the eventual production of specific immunoglobulins. It is highly probable that a portion of the injected heterologous erythrocytes undergo immediate lysis upon reaching the spleen (33), while phagocytes present in the red pulp and in the marginal zone take up the foreign cells (28, 46), and, within several hours, destroy most of their immunogenic activity (Fig. 7A). The fact that immunogens, isolated 1 hr after antigen injection, are capable of stimulating both primary and secondary immune responses seems to indicate that, at this time, antigenic fragments have not as yet been fully degraded by macrophages, or that immunogenic materials have possibly been retained on the surfaces of these tissue phagocytes (62), or that immunogens have become specifically attached to specialized cells with immune receptors specific for sheep erythrocytes (54). It is not clear at present exactly when and how the tertiary structure of an immunogen is presented to the antigen-sensitive cell. Nevertheless, by 24 hr when sheep cell immunogens, capable of inducing secondary immune responses, are primarily associated with the membranes of lymphoid cells (Bachvaroff and Franzl, Fed. Proc. **29**:825, 1970), antigen-binding thymus-derived small lymphocytes of the spleen become specifically stimulated to give rise to large pyroninophilic cells (39, 40). Some of these cells, which have previously been described in the marginal zone of the mouse's spleen (23, 36), then appear to differentiate into small lymphocytes (40), possibly the very same, early-primed memory cells found in the spleens of mice (52; T. H. Carter and R. E. Franzl, Fed. Proc. **27**:493, 1968) and rabbits (9) on the second day after giving sheep RBC, whereas the remainder later transform into antibody-producing cells (23, 36). At this time, 2 days after antigen injection, the spleen contains an appreciably lower content of sheep RBC immunogens than on days 1 and 3 (Fig. 7A), an observation further accentuated by the striking differences between 1- and 3-day immunogenic "L" fractions in their susceptibilities to inactivation by antiserum and by a surface-active agent (Table 4). Both findings indicate that changes in the phyiscal and serological properties of retained immunogens are taking place during the period from 1 to 3 days after antigen administration. Furthermore, the changes in the immunogen on the third day, resulting from its possible interaction with the actively produced immunoglobulin M (IgM) antibody (Fig. 7B), might also reflect a relocation of reactive material, originally associated with cells of the marginal sinus, to the antibody-dependent sites in lymphoid follicles (reviewed in 24), in a manner analogous to the reported migration of labeled flagella in the rat spleen (28). The resulting follicular localization of an immunogen of high activity might well be required to stimulate the precursors of IgG anti-sheep hemolysin-producing cells, which make their appearance on day 4 in the mouse. However, it still remains to be proven whether a specialized localization of biologically active immunogens in the spleen can be identified with the postulated immunological activities of labeled antigen localized in specific areas of lymph nodes of the rat (2). The sudden drop in sheep cell immunogenicity on day 4, concomitant with the peak of IgM antibody production (Fig. 7A and B), suggests a formation of specific immune complexes, analogous to those which similarly inhibit immune responses in rabbits (47), which, by effectively decreasing the concentration of immunogenic determinants, result in the early shutting off of IgM production in the spleen. The continued rise in the titer of serum hemolysins after this time (Fig. 7C) undoubtedly represents the continued production of IgG immunoglobulins by long-lived cells.

In line with the above considerations, it is reasonable to assume that the relatively long-lived immunogenic stimulus retained by the spleen permits an efficient transmission of antigenic information to antigen-sensitive cells, and favors the subsequent differentiation and proliferation of these cells into the specific populations which establish immediate and long-term immunity in the organism.

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