Observations on the Kinetics of Hemolytic Antibody Response by Localized Hemolysis in Gel over Frozen Sections of Mouse Spleen. (32372)

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The single cell method of Jerne and Nordin (1) and Ingraham and Bussard(2) allows quantitation of the immune response to various antigens by single lymphoid cells(3,4,5). Recent modifications of this method involving thin frozen sections of spleen instead of isolated single cells have also been used to investigate the immune response(6,7,8,9). This report is concerned with observations on the kinetics of the hemolysin response in mice to a single injection of sheep erythrocyte when measured by a frozen section technique.

Materials and methods. Animals and immunization schedule. Balb/C female mice weighing 20-25 g from the animal production section of the National Institutes of Health were immunized intraperitoneally with 0.5 ml of washed 10% sheep erythrocytes (SRBC) in saline. Non-immunized mice served as controls. On appropriate days, 4-10 experimental and control mice were bled by cardiac puncture and sacrificed by cervical dislocation. The spleen was removed from each animal and cut transversely to furnish proximal and distal havles of similar length.

Frozen section technique. Proximal halves of spleens from immunized and non-immunized mice were frozen over dry ice and mounted on object discs so that 10 μ sections could be cut parallel to the ventral surface of the organ. Five such sections were cut from the middle portion of each spleen at 80-120 μ intervals and were placed on glass slides to dry. Several drops of 0.5% agarose (Bausch and Lomb, Inc., Rochester, N. Y.) containing 0.8% SRBC and 20% reconstituted guinea pig complement (Baltimore Biological Laboratory, Baltimore, Md.) were immediately dropped onto the dried sections from a 5 ml pipette. After allowing the agar to harden, slides were incubated for one hour at 37°C in 100% relative humidity. The localized areas of hemolysis which appeared in the agar-SRBC overlay were termed "foci." Sections were examined and foci were enumerated with the aid of a dissecting microscope at 7× magnification. The circular areas of hemolysis were counted regardless of diametric size and each was scored as a unit. Larger foci were often irregular in shape due to overlapping of individual foci. In such cases, each lobe of an irregular focus was scored as a separate unit.

The dependence of foci formation on complement was established by either omitting it from the agar or adding it after heat inactivation or ethylene-diaminetetraacetic acid inhibition. The possibility that foci were formed by serum antibody in the tissue was ruled out by the observation that no such localized hemolysis occurred over frozen sections of liver from immunized mice.

Jerne technique. A modified Jerne technique was used to determine the number of individual plaque-forming spleen cells (PFC) (1). Modifications of this technique included the use of Ham's F-12 medium(10) in place of Eagle's medium and the substitution of 0.5% agarose(11) for Difco agar. Guinea pig complement diluted 1/10 was used as the complement source.

The number and distribution of PFC in proximal and distal spleen halves were determined in immunized and non-immunized mice. The release of cells from spleen tissue was effected by gentle homogenization with a loose fitting teflon pestle in a test tube, and the number of cells in each suspension was determined by hemocytometer counts. Cell concentrations were adjusted so that between 5×10^5 and 5×10^7 cells were plated on appropriate days. In further experiments, the Jerne technique was used to assay only the distal spleen half.

Serum hemolysin determination. Circulat-

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ing hemolysin titers were determined by the Takatsy microtiter technique(12) employing 2-fold serum dilutions beginning at 1:2 and utilizing 50% hemolysis as the end point.

Topographic relationship of foci to splenic red pulp and lymphatic nodules. Spleen sections over which foci had developed were examined under a dissecting scope at 25× magnification. The outlines of foci were scratched on the reverse side of the slide with a diamond needle. The agar was then removed by first dipping the slide in 80°C water for several seconds and then carefully reflecting it off the tissue with a needle. The spleen section was fixed in 10% formalin and stained with hematoxylin and eosin. Although cellular detail was lacking due to the manner in which the tissue had been handled, architectural features such as the red and white pulp, the lymphatic nodules and the germinal centers were easily seen.

Results. The kinetics of the primary hemolytic antibody response to SRBC were studied in individual spleens using both the frozen section technique (FST) and the Jerne single cell method. The foci response was determined in frozen sections from proximal spleen halves while the PFC response was determined in distal spleen halves. In order to compare the results obtained from either spleen half, it was necessary to determine if a similar distribution of PFC existed in the two halves. Data shown in Table I are representative of results from groups of 10 mice which were assayed for the number of PFC in each spleen half at different times after immunization. The data represent the number of PFC per 10^8 cells. Total cell numbers ranged from 0.81×10^8 to 1.09×10^8 in the proximal spleen half, and from 0.88×10^8 to 1.69×10^8 in the distal spleen half. The results obtained from immunized mice suggested that there was a similar distribution of PFC in each spleen half.

TABLE I. Number of PFC per 10^8 Cells in Each Half Spleen from Immunized and Non-Immunized Mice.

Days after immunization	Animal	Proximal half spleen	Distal half spleen
Nonimmunized	A	14	11
	B	12	16
	C	26	33
4	A	35800	35800
	B	39500	35900
70	A	99	95
	B	154	141
	C	78	88

The appearance of foci over sections of spleen from non-immunized mice is shown in Fig. 1. Generally, the small hazy type of foci (A, Fig. 1) predominated, but occasionally large clear foci were seen in which complete hemolysis had occurred (B, Fig. 1). Serial sections showed that small foci (approximately 0.1 mm in diameter) were rarely peripheral areas of large foci which resulted because of sectioning artifact. Large foci were usually 3 to 5 times the diametric size of small foci. Based on 26 non-immunized mice, the range of "background" foci was 0 to 15 per section of proximal half spleen with a mean and standard error of the mean (SEM) of 6.0 ± 1.6. The difference in numbers of foci over control and immunized spleens can be appreciated if Fig. 2 is observed. Throughout the experimental period, foci over sections of immunized spleen were predominantly of the large type. The various intensities of lysis which are seen in numerous foci were predominantly the result of sectioning at different planes through antibody-releasing sites. The number of foci which occurred over sections of spleen from immunized mice rose rapidly between days 1 and 4 as is indicated in Fig. 3. Between

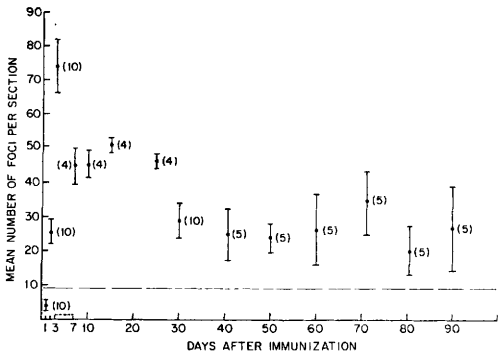

FIG. 3. Rise and fall in number of foci per section of proximal spleen halves. Broken line above the abscissa represents average number of background foci per section plus 2 SEM (6.0 ± 3.2). Dotted line between days 3 and 7 represents occurrence of confluent hemolysis. Each point is a mean ± 2 SEM number of animals used to determine each mean in parenthesis.

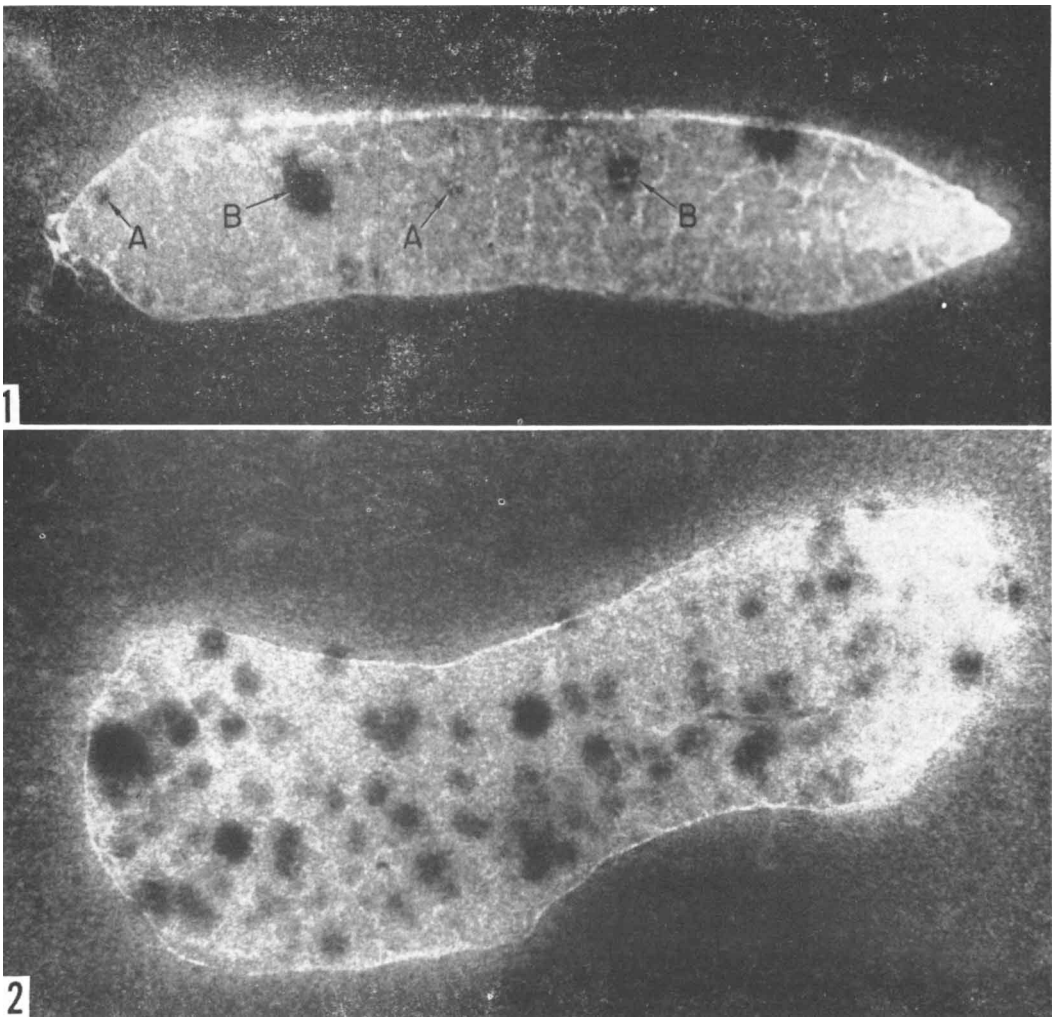

FIG. 1. A section of whole spleen from a non-immunized mouse overlaid with SRBC, agar, and complement showing foci. The foci present are generally of 2 distinct types. "A" represents the small hazy type of foci; "B" the larger clear type of foci.

FIG. 2. A section of whole spleen overlaid with SRBC, agar, and complement showing the predominantly large type of foci. This section was from a spleen 10 days after immunization and shows the large number of foci present at this time.

days 3 and 7, complete coalescence of foci occurred and practically all SRBC overlying the spleen section were lysed, thus eliminating individual foci. This period corresponded to the time when maximum PFC were present in the spleen and highest hemolytic antibody titers existed in the serum(1). After 3 to 7 days post immunization the amount of hemolytic antibody released in spleen sections decreased and allowed individual foci again to be perceived and enumerated. Although the foci response dropped steadily after reaching peak activity, a smaller subsequent peak was suggested by the data between days 10 and 30 which corresponded temporally to the onset of PFC releasing 7S antibody(13,14). In general, however, the results of foci quantitation illustrated that the number of foci per section decreased to a plateau level after declining from maximal activity. Such plateau levels persisted above control levels for at least 90 days after immunization. Additional experiments have shown that foci activity remained significantly elevated for 113

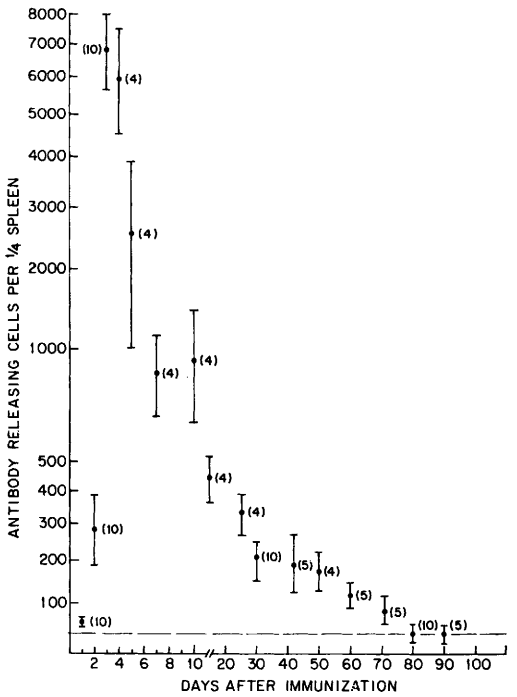

FIG. 4. Rise and fall in number of PFC from distal spleen halves. Broken line above abscissa represents the mean of background PFC plus 2 SEM (24.6 ± 8.1). Each point is the mean ± 2 SEM as determined from number of animals indicated in parenthesis.

days after immunization, however, at 210 days foci activity had declined to "background" levels.

Data based on the single cell response obtained from distal spleen halves are presented in Fig. 4. A rapid rise and fall of PFC occurred between days 1 and 7 post immunization. The number of PFC continued to drop rapidly beyond day 7 until background levels were reached before the end of the experimental period. The number of "background" PFC ranged from 2 to 80 per distal half spleen. The mean and SEM based on 26 non-immunized mice was 24 ± 4.1

The results of serum hemolysin titers indicated maximum levels at day 5 after immunization. No differences between immune and control sera could be detected beyond 40 to 50 days post immunization.

Attempts to study the topographic relationships of foci to splenic red pulp and lymphatic nodules showed that in all cases where individual foci were examined, hemolytic activity was closely associated with the peripheral portion of the lymphatic nodule as well as the juxtaposed red pulp. Whether the cells which released such hemolysins were located in the red pulp or the lymphatic nodule itself could not be determined.

Discussion. The data presented herein indicate that, in general, the kinetics of the foci response in frozen sections are similar to the PFC response. Differences which exist between the two parameters are probably due to the greater sensitivity of the FST. Such is thought to be the case in light of the suggestion that foci are the result of a concerted release of antibody by clusters of cells(6,7). The persistence of foci activity during the absence of significant PFC activity could be explained on the basis of larger amounts of antibody present to effect discernible hemolysis. Besides the virtuous arrangement of cells in clusters to provide concerted antibody release, the preparation of frozen sections *per se* might effect the release of stored antibody from cells, for cellular release of this antibody has been attributed to altered cellular permeability(15). If such is the case, cells which contain stored antibody could go undetected in the more physiologic PFC system.

Because of the greater sensitivity possible with the FST as opposed to the PFC method, and because suspect antibody containing cells need not be removed from their resident tissues, the FST may be better suited than the PFC method for the detection of cells containing specific antibody in tissues.

Our observation regarding the approximation of foci to lymphatic nodules may have significance in terms of the secondary immune response. Nossal(16) has shown that lymphatic nodules of animals which had previously experienced a primary immune response had a high specific affinity for homologous antigens during the secondary response. This affinity was apparently the result of opsonizing antibody produced during the primary response which had localized in the lymphatic nodules. If such is the case in the present experiments, foci which develop over lymphatic nodules could be due to a hemolytic activity of the same or similar

types of localized antibody referred to by Nossal. The possibility that localized hemolytic antibody may persist in lymphatic nodules as a result of primary immunization and that such antibody is detectable by the FST may allow one to predict the readiness of the immune mechanism to experience a secondary response.

Summary. The kinetics of the hemolytic antibody response in mice to a single injection of sheep erythrocytes were determined by quantitating the number of hemolytic foci which appeared in an agar-erythrocyte overlay on thin sections of mouse spleen. After immunization, the number of foci rose to a peak and then declined gradually to levels which persisted through 113 days. At 210 days after immunization, foci activity had merged with control levels. On the other hand, the plaque-forming cell method of Jerne showed an increase in the number of plaque-forming cells until a peak was reached with a subsequent rapid decline to control levels 70-80 days after immunization. It is concluded that the frozen section technique is a sensitive method for the discrimination and enumeration of cells containing specific antibody in tissues.

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Effect of Transplanted Tumor and Various Agents on Liver Regeneration During Pregnancy. (32373)

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In a previous study, the effect of agents which stimulate or inhibit the extent of liver regeneration in partially hepatectomized animals was determined in rats with Walker tumor transplanted directly into the surviving liver at surgery(1). The presence of tumor tended to normalize the regenerative rate in groups treated with several of the agents and except for the depressant action of nicotinamide, tumor growth was little affected in their presence. The current experiments were devised to ascertain the simultaneous effect of pregnancy, a condition under which liver weight restoration is accelerated (2,3) and transplanted tumor on liver regeneration and lesion growth as well as of the combined influence of pregnancy and depressant or stimulating agents on the hepatic process.

Materials and methods. The agents were incorporated into Rockland rat meal on a weight basis, the respective compositions being the same as those employed previously (1): acenaphthene (0.25%; Gesellschaft für