THE FATE OF INJECTED PARTICULATE ANTIGENS IN RELATION TO THE FORMATION OF ANTIBODIES

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In earlier papers of this series it was shown that following the injection of an antigen into the pad of the rabbit's foot there was marked increase in the size of the regional lymph node and in the output of lymphocytes by that node. At the same time it was found that antibodies to the antigenic material injected could be identified in an aqueous extract of the lymph node as well as in lymph collected from its efferent lymphatic vessel (1). Later it was shown that the lymphocytes in such lymph contained a higher concentration of antibody than the lymph plasma surrounding them, and that this difference was greatest at the time of the highest rate of production of antibodies. The results of several types of experiments indicated that the antibody found within the lymphocytes was not absorbed or concentrated from the environment but presumably arose within the lymphocyte (2).

More recent experiments have shown that antibodies are not found in the granulocytes or macrophages of inflammatory exudates at the time of greatest antibody production, when both exudate and blood serum contain antibodies. This evidence is a basis (3) for the belief that granulocytes or macrophages are not the site of the synthesis of antibodies.

Since the lymphocyte is not a phagocytic cell, and yet the evidence points to synthesis of antibody by that cell, it seems probable that the relatively large unit of particulate antigen used in the experiments, and ordinarily encountered in infections, must undergo a change in structure such that it will be in a soluble form available to the lymphocyte. Accordingly, a study was instituted of the developments in the local and lymphatic tissue in the period between the injection of the antigenic material and the first appearance of antibodies in the lymph.

Methods

The system studied was the same as that used in the earlier papers of this series: Antigens were injected into the pad of the rabbit's foot in quantities of 0.2 cc. At different intervals thereafter the popliteal lymph node, which drains the area of infection, and its efferent lymphatic were exposed. Lymph was withdrawn from the efferent lymphatic vessel, and the lymph node and the site of the injection were excised.

The antigens used were sheep erythrocytes, washed in saline solution and packed in the

centrifuge, and dysentery organisms, *Shigella paradysenteriae* Flexner Z 911 A, grown and prepared as described in an earlier paper (3). The latter were injected in a concentration of 5 mg. of dry weight per cc.

Lymph nodes were excised as formerly, weighed, and ground with one volume of fine sand and three volumes of saline solution. The resultant suspension was centrifuged until the supernatant fluid was clear. This fluid was considered lymph node extract 1:4.

Extracts of the tissue at the site of injection were prepared as follows: The skin over the pad of the foot was incised and reflected, exposing the tendons beneath it. The four tendons were cut and reflected, and the underlying tissue, into which the antigen had been injected, was excised. This was extracted in the same manner as the lymph node.

Lymph was collected and the tests for antibody were performed as described in the earlier papers (2, 3). The tests for concentration of antigen-specific soluble material were done as follows:—

(a) Inhibition of hemolysis of sheep erythrocytes: Dilutions of the clear tissue extract or of lymph in amounts of 0.2 cc. were mixed with 0.1 cc. of rabbit anti-sheep hemolysin which had been diluted to contain 1.5 units per 0.1 cc., usually 1:1800. After 20 minutes of incubation at room temperature 0.2 cc. of a mixture of 5 per cent washed sheep cells and complement was added to each tube. After 30 minutes of incubation at 37°C. the extent of hemolysis was determined in each tube. The guinea pig serum used for complement was employed in the high concentration of 1:10 in order to obviate anticomplementary effects of tissue extracts. The spontaneous occurrence of rabbit anti-sheep hemolysins in the animals used was controlled by preliminary tests.

(b) Inhibition of agglutination of dysentery organisms: Twofold dilutions of the material to be examined were made, using 0.4 cc. amounts. To each dilution was added 0.1 cc. of anti-serum which had been prepared by the immunization of rabbits with alcohol-killed organisms of the strain providing the antigen used. After 20 minutes of incubation at room temperature 0.1 cc. of a saline suspension of alcohol-killed organisms containing 0.5 mg. of bacterial protein per cc. was added to each tube. The tubes were then incubated for 1 hour at 37°C. and allowed to stand overnight in the refrigerator. The degree of agglutination was read according to the bacterial cells on the bottom of the tube, as described elsewhere (3).

(c) Complement fixation: Twofold dilutions were made of the material to be examined, using 0.4 cc. amounts. To each tube was added 0.1 cc. of saline solution containing guinea pig serum, for complement, and antidysentery serum. After 1 hour of incubation at 37°C. each tube received 0.1 cc. of sensitized cells, prepared as a 1 per cent suspension of sheep erythrocytes in a saline dilution of rabbit anti-sheep-erythrocyte serum, the final dilution of serum being 1:500. After another half-hour of incubation at 37°C. the degree of hemolysis was read.

The hemolysin was used in excess. The concentration of guinea pig serum was such that each tube received 1.5 times the amount of complement necessary for complete hemolysis of the erythrocyte suspension used, in blank control tests. The concentration of the antidysentery serum used was optimal, as determined by preliminary tests against the experimental materials, both antigen and serum being used in various dilutions. The antidysentery serum chosen was, of course, of sufficiently high titer that no anticomplementary effect could be detected even at much higher concentrations than the optimal one used in the test. As to anticomplementary properties of the tissue extracts and lymph, these were controlled by duplicate tubes in which the antidysentery serum was omitted. Finally, since low titers of antibodies which react with dysentery antigen are found in normal rabbits, the possible interference of such material with our experimental results was avoided by preliminary bleedings in the group of rabbits used.

All additions of reagents were made by automatic pipette, with calibrations checked at each test.

EXPERIMENTAL

Inhibition of Hemolysis of Sheep Erythrocytes.—The first series of experiments was done with sheep erythrocytes. These were prepared and injected in the same manner as in earlier studies (1). After varying periods ranging from 12 hours until the appearance of antibodies in the local lymphatic tissue, efferent lymph from the regional lymph node was collected and the injected foot pad and the node were excised for extraction. These materials were analyzed for antibody content and for the presence of soluble material of the same immunologic specificity as the cell injected. For brevity, the concentration of such antigen-specific soluble material is indicated in the tables as "antigen titer."

TABLE I
Concentrations of Antigen-Specific Soluble Material and of Antibody in Local Tissues and Lymphatic System in the Period Ending with the Appearance of Antibodies
Sheep R.B.C.: Inhibition of Hemolysis

Days	No. of specimens	Antigen titer* geometric mean			Antibody titer geometric mean			Serum antibody titer
		Foot pad	Lymph node extract	Lymph	Foot pad	Lymph node extract	Lymph	
$\frac{1}{2}$	4	192	20	1	0	0	0	0
1	16	512	16	3	0	0	0	3
2	8	480	16	2	0	0	0	0
3	4	288	16	1	0	0	0	4
4	4	96	0	0	0	48	16	12
5	2	80	0	0	0	96	48	64

* The term "antigen titer" is used, by analogy with antibody titer, to express the highest dilution of the specimen at which inhibition of hemolysis could be demonstrated in the volume used in the test.

It is used in the same way in Tables II and IV.

Table I shows the results of thirty-eight experiments by hemolysis tests and by inhibition of hemolysis. On following the sequence of determinations performed on extracts of the tissue at the site of injection it is seen that antigen-specific soluble material appears within a few hours. The concentration of this material rises to a higher level for about 2 days, and then falls off gradually.

In the tissue of the lymph node the antigen-specific soluble material also appears very soon and remains at a lower concentration until the time of appearance of antibodies. The lymph emerging from the node shows a similar course of events, in somewhat lower titer. At the 4th day no antigenic material can be detected in the lymphatic system. At that time the expected finding of antibody in the local lymphatic system and in the serum of the animal is made, while the injected tissue still contains antigen-specific material.

Inhibition of Agglutination of Dysentery Organisms.—More extensive experiments were done with killed dysentery organisms as the particulate antigenic

material. In one such series of twenty-eight experiments 1 mg. of the organisms was injected into the foot pad and the materials to be analyzed were collected $\frac{1}{2}$, 1, 2, and 3 days thereafter. The test for antigen-specific material was in this series the inhibition of agglutination. The pattern test used in these determinations presents a distinct advantage, in addition to its greater sensitivity, in that the absence of agglutination, corresponding to the presence of inhibiting substance, is indicated by a characteristic central button of unagglutinated bacterial cells (3). Thus the findings of antigen-specific material, as shown in the data, rest, not on the failure to observe agglutination in a given tube, but on the observation of a distinct geometric form.

Table II shows the results of a typical experiment.

It will be noted that the same sequence of events takes place as in the case of the sheep erythrocytes, although some quantitative differences are found in

TABLE II
Concentrations of Antigen-Specific Soluble Material and of Antibody in Local Tissues and Lymphatic System in the Period Ending with the Appearance of Antibodies
Dysentery Organisms: Inhibition of Agglutination

Days	No. of Specimens	Antigen titer geometric mean			Antibody titer geometric mean			Serum antibody titer
		Foot pad	Lymph node extract	Lymph	Foot pad	Lymph node extract	Lymph	
0	6	0	0	0	0	0	0	6
$\frac{1}{2}$	8	240	44	20	0	0	0	8
1	8	256	22	4	0	0	0	6
2	8	80	6	2	0	0	0	32
3	4	32	0	0	0	96	32	768

concentrations and times. Within a few hours, relatively high titers of antigen-specific material were found in all the tissues examined. The concentration of this material fell off quite soon in the lymphatic system and, after 2 days, in the injected tissue as well. After 3 days, when antibody was found in the lymphatic system, there was of course no antigen detectable, but residual antigen-specific material could still be found at the site of injection.

Although the definite pattern associated with inhibition of agglutination and the inclusion of full sets of control tubes in each test seemed to render the interpretation of the results unequivocal, the following experiment was done to test the conclusion that a true immunologic neutralization was being observed. Representative extracts of injected tissue and of lymph node excised 1 day after injection of dysentery organisms were diluted in steps of two and tested against various dilutions of the antidysentery serum used in the inhibition experiment reported above. The test for inhibition of agglutination was carried out as for the experiment of Table II.

The results of such a two-way titration are shown in Table III.

The readings after the organisms had been allowed to settle overnight were made as for the other experiment in this study: "4" denotes a membranous sediment of agglutinated organisms, which on shaking breaks up into coarse floccules; "3" denotes granules spread over the bottom of the tube, which can easily be shaken up into the clear medium above; "2" denotes a barely visible sediment spread over the bottom of the tube, and no visible granules on shaking; "1" denotes a central button of unagglutinated organisms, its circumference rendered indistinct by a relatively few outlying clumps of agglutinated cells; and "0" indicates a sharply bordered button of bacterial cells among which presumably no agglutination whatever has taken place. The last is taken to correspond to complete inhibition; in the tubes read as "1" the inhibition was probably almost complete.

On examination of Table III it will be seen that the border separating readings of "0" and "1" from those of "2," "3," and "4" falls in an essentially straight line inclined at 45°, in the case of both foot-pad extract and lymph node extract. This is what would be expected as the consequence of optimal proportions in

TABLE III
*Correlation of Dilutions of Serum and of Antigen-Specific Soluble Material
Dysentery Organisms: Inhibition of Agglutination*

Serum	Foot pad....	32	64	128	256	512	1024	2048	Lymph node...4	8	16	32	64	Control
128	4	4	4	4	4	4	4	4	1	4	4	4	4	4
256	4	4	4	4	4	3	4	4	1	2	4	4	4	4
512	0	3	4	4	4	4	4	4	1	1	2	3	4	4
1024	0	0	3	3	3	3	3	3	1	0	0	1	3	3
2048	0	0	0	2	3	3	3	3	0	0	0	0	3	3
4096	0	0	0	1	2	2	2	2	0	0	0	0	0	2

true immunologic neutralization. The row in which the serum was used in a concentration of 1:2048 corresponds to the routine technic used in all the experiments to detect inhibition of agglutination in this study.

Complement Fixation.—In other experiments the preparations from the injected rabbits were tested for the concentration of antigen-specific soluble material by complement fixation. The results of a series of such experiments are shown in Table IV.

The results of the complement fixation tests are interesting in comparison with those of the inhibition tests shown in Table II. They offer a confirmation, by a different reaction, of the fact that the material found in clear extracts of the tissues involved and in the lymph has the same immunologic specificity as the particulate antigen injected. Additional confirmation is offered by the general trend of titers, which is quite similar.

Controls.—The fine poisoning of these tests, the small quantities of materials involved, and the low titers frequently obtained render particularly advisable an account of the controls.

The usual controls were, of course, set up in each test. These included "positive controls" in which saline solution was substituted for the dilution of material tested, allowing the specific antiserum to act on the cell species involved, tubes containing the latter alone, and specificity controls in which were used corresponding extracts derived from animals injected with the other antigen. In the case of the inhibition of agglutination the test was so satisfactorily poised that even in the test for antigen in lymph it was possible to detect the appearance of antibodies, at the beginning of the 3rd day, by the appearance of "4+" bacterial sediments, as compared with the very fine granular ones in the control tubes containing only antiserum and cells.

Additional precautions were taken in the complement fixation tests. As noted above, the antidyentery serum used showed an optimal complement fixation titer far greater than the highest one which showed anticomplementary activity against the 1.5 units of complement employed. This was particularly desirable in order to avoid possible summation of undetected anticomplementary effects of the serum and of the tissue extract. As to the latter,

TABLE IV
Concentrations of Antigen-Specific Soluble Material in Local Tissues and Lymphatic System in the Period Prior to the Appearance of Antibodies
Dysentery Organisms: Complement Fixation

Days	No. of specimens	Antigen titer geometric mean			Control preparations (4) Injected with R.B.C.		
		Foot pad	Lymph node extract	Lymph	Foot pad	Lymph node extract	Lymph
0	6	0	0	0	0	0	0
$\frac{1}{2}$	4	256	48	12	0	0	0
1	16	284	32	8	0	0	0
2	8	192	8	6	0	0	0
3	4	96	0	0	0	0	0

each test was set up with a complete set of duplicates for anticomplementary controls of the tissue extract, rather than one tube containing the largest amount used. Low titers of such anticomplementary effect were, in fact, often found in the lymph as well as in tissue extracts.

Physiologic Significance.—In addition to these technical controls certain preparations were included in the larger tests to show whether the material giving antigen-specific reactions was the result of a physiologic process, not an artefact in the preparation of the materials.

In order to learn whether the soluble antigen-specific material demonstrable in the tissues was the result of the sand-grinding of tissues into which whole dysentery organisms had been injected or carried *via* the afferent lymph, a set of experiments was done, duplicating that summarized in Table II, in which the tissues were merely well minced with strabismus scissors and then extracted with saline solution. The results were similar to those shown in Table II.

As bearing on whether the specific soluble material was the result of some physiologic process initiated by the injection, the procedure of injection and excision was repeated in six experiments, except that the excision of foot pad and lymph node was carried out immediately after injection. In all of these tests the results were entirely negative; no antigen-specific material could be identified in the extracts. This set of experiments also furnished additional

evidence that the specific soluble material does not arise by grinding of organisms imbedded in tissue.

Finally, as additional evidence that some physiologic process was involved, it was shown that the mere maintenance of alcohol-killed dysentery organisms, as prepared for these experiments, at a temperature of 37°C. for some time did not give rise to antigen-specific soluble material in their immediate environment by lysis of the organisms. A sterile suspension of alcohol-killed organisms, similar to the one injected, was kept in the incubator at 37°C. for 6 days, or twelve times the period after which specific soluble material is easily demonstrable following injection into the tissues. The supernatant was then tested by complement fixation. The result was entirely negative; there was absence of hemolysis only in the first tube, or 1:4 dilution, corresponding to an anticomplementary effect in the control tube.

DISCUSSION

The implications of the experimental findings would appear to be as follows: Following the local injections of particulate antigenic material, the particles are subjected to a physiologic process which results in the liberation of smaller soluble particles. The latter retain groupings which are immunologically characteristic of the original antigenic material. They are carried by the lymph stream to the regional lymph node where they come in contact with lymphatic tissue. In this way they stimulate the antibody-synthesizing mechanism of lymphatic tissue.

It may well be that phagocytosis and intracellular digestion of the antigenic particles are involved in the early stage of preparation of injected material for the lymphatic tissue. If that is the case then these experiments furnish the connecting link between the classical "blockade" experiments, on which the reticulo-endothelial theory of antibody formation is based, and what we now know of the function of the lymphocyte in the formation of antibodies.

A word seems in order on the relations of quantity and time to the amounts of antigen-specific material and antibody found in the injected tissue and its regional lymph node. It seems entirely probable that many factors are involved: the amount of specific and non-specific protein injected; the degree of tissue irritation caused by the material injected; qualitative differences in the response of various cell types to the irritation and, finally, the effectiveness of the specific antigenic groupings involved as stimuli to antibody production. The larger the amount of material injected, the greater and more prolonged should be the concentration of antigen-specific material at the site of the injection, and secondarily in the lymphatic system. If an antigen is relatively more irritating to the tissues, perhaps particularly in stimulating an outpouring of macrophages, one might expect a higher concentration of antigen-specific material, but one of shorter duration. Again, in the case of an antigen particularly effective in stimulating the production of antibodies, the rising time curve of antibody concentration would sooner intersect the curve of antigen-specific material concentration so that the efferent lymph would show antibodies relatively soon.

The manipulation of the variables mentioned and an estimation of their effects would require a great expenditure of painstaking immunologic experimentation, and it does not lie within the scope of this paper. However it is likely that the differences in time relation between Tables I and II are attributable to the fact that in Table II we are dealing with a lighter suspension of cells of a type which is more effective as an irritant and as an antigen than is the sheep erythrocyte. Again, in drawing conclusions from relative titers in different tissues at any given time, it must be remembered that we are dealing with a complex dynamic system. The titer in the extract of injected tissue, for example, represents a difference between the rate of liberation of antigen-specific soluble material from the injected antigen, and the rate of its removal into the lymph stream. Again, the titer of antigen-specific material in the extract of lymph node at any moment is the resultant of at least three factors: the rate of arrival of this material in the afferent lymph; the rate at which it is utilized in the node, and the neutralization of this material by the increasing amounts of antibody formed before the latter is yet detectable. The titers do, however, indicate the sequence of immunologic events, especially when they are followed in a given tissue at different times.

Not all of these variables, however, may be of importance in determining the final antibody concentration attained in the serum. It is quite probable that when antigen-specific material reaches the regional lymph node in such high concentration that some of it passes through unchanged, the excess, which is detectable in the efferent lymph of that node, serves to stimulate antibody formation in the next lymph node in its course. If the process is so acute or severe that antigen-specific material enters the blood in effective quantity after passing all the nodes in its path it may conceivably stimulate distant organs to antibody production.

SUMMARY

Earlier studies have shown that the injection of antigenic material into the pad of the rabbit's foot is followed by the appearance of antibodies in the regional lymph node, in lymph coming from that node, and especially in the lymphocytes present in such efferent lymph. In the present work the fate of particulate antigenic material has been investigated during the period between its injection into the foot of the rabbit and the appearance of antibodies in the regional lymphatic tissue.

It has been found that soluble substance of the same immunologic specificity as the antigenic material injected can be identified in extracts of the injected tissue and of the regional lymph node, and in the efferent lymph from that node. The concentration of this soluble material falls off slowly in the injected tissue in the course of the few days following the injection. It falls off quickly in the extract of the lymph node and in the lymph itself, and its disappearance is succeeded by the appearance of antibody.

Evidence is presented that the immunologically active substance is derived from the injected antigenic material by a physiologic process, and this process is discussed as the means by which antigens, originally comprised in cells, are made available to the lymphocyte.

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