THE FORMATION OF AGGLUTININS WITHIN LYMPH NODES

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In human skin the superficial lymphatic plexus is so rich that every scratch or puncture wound serves to rupture some of the minute lymphatic capillaries (1). Peripheral lymph flow is far more rapid than is generally supposed (1), and dye substances injected intradermally are carried to neighboring lymph nodes in a few minutes, even in a resting limb (1). Work on the physiology of lymphatics in the ear of the mouse (2) has shown that lymphatic capillaries remain open many hours after an incision. Whenever the continuity of the skin is broken there exists therefore a ready route for infection. Along the path of the infection, between the open lymphatic capillaries of the skin and the entrance of the larger channels into the blood stream, stand the regional lymph nodes; and when such infection occurs with or without lymphangitis they become enlarged and painful. A reaction of lymph nodes to infection is recognized in a multitude of diseases,—as *e.g.* in plague, in typhoid and frequently in tonsillar infection. Pathogenic bacteria carried on the lymph stream are often arrested in the glands through which this stream passes, with result that the infection travels no further.

It seems possible that within the lymph glands there may occur some formation of antibodies to antigens arriving by the lymph stream. The opportunity to test this conception has presented itself to us incidentally to work on the lymphatics of the ear of the mouse. These were rendered visible by dyes and it was found that the extremely rich plexus of superficial lymphatic capillaries in the skin (3–5) drains into one or two common trunks at the base of the ear, which convey the dye to a cervical lymph node, or occasionally a small group of nodes, lying superficially along the margins of the

thyroid gland. In some preliminary experiments lymphangitis was induced by intradermal injection of typhoid bacterins[1] upon 2 successive days, in the right ears of mice. 4 days to a week later the animals were autopsied and examined. The injections were found to have been largely intralymphatic, and in addition to lymphangitis in the ears there was a great enlargement of the cervical lymph nodes. This way of bringing antigen to the lymph nodes was utilized to find whether these form agglutinins for bacteria.

Methods

Mice of 25–35 gm. body weight were used and approximately 0.02 cc. of the various antigens, to be described below, were injected intradermally into the ears. The injections were repeated at varying intervals as the conditions of each experiment required. To assure ourselves that the injections were truly intradermal, they were carried out beneath the binocular microscope with the mouse under light ether anesthesia and the ears spread on porcelain plaques (3).

At varying intervals following the injections the materials to be tested for antibody content were obtained and extracted in the following manner. The mouse anesthetized with ether or luminal was placed on its back with fore legs slightly elevated. The neck was painted with paraffin oil, and by means of sterile scissors the skin was cut in a T-shaped incision from chin to sternum and then at right angles to this over the axillary vessels on each side. With other sterile instruments the connective tissue was dissected free as the skin was reflected. The procedure disclosed a small superficial lymph node which lies along the cervical vessels near the masseter muscle, receiving lymphatics chiefly from the tongue, and another node or group of nodes lying deeper and more laterally, along the margins of the thyroid gland and receiving lymphatics from the ear. The latter were usually found much enlarged, and were easily shelled out by dissecting away the capsules, scarcely touching the nodes themselves. The mice were then exsanguinated from the carotid artery and the blood specimens pooled for serum.

After weighing the nodes they were aseptically ground with sand, with gradual additions of either sterile 0.2 per cent saline solution or a mixture of equal parts of the latter with glycerine, as the experiments required. In a few experiments only 15 cc. of fluid was used per gram of fresh lymph node material. In most at least 31 or 63 cc. were employed, or more when it was felt the concentration of agglutinins was high. In our first experiments only the glycerine-saline mixture was used, for earlier workers (6, 7) have reported it to be the best extracting fluid for hemolysins. In the majority of experiments, however, saline solution only was employed and the tests were done immediately. In a few experiments the spleens

[1] New York State Board of Health Typhoid Vaccine.

of the animals were also ground and extracted. The extracts were centrifuged for an hour at 2500 revolutions a minute in sterile 15 cc. centrifuge tubes, and at times separation of the fatty pellicle was aided by first allowing the tubes to stand in the ice box. The clear central portion of the fluid was removed from the tubes with Wright's pipettes, thus avoiding the fatty pellicle and sediment. The fluid thus obtained was centrifuged again for half an hour and the process was repeated for further clearing. If not yet clear the fluid was diluted further and again centrifuged. It is to be noted that these extracts were made as dilute as possible to rule out non-specific flocculation which might occur during the performance of agglutination reactions with concentrated tissue extracts. No doubt we lost much valuable material in the repeated centrifugations.

Agglutination Reactions.—The cleared lymph node or spleen extracts and the sera of the animals, cleared of blood cells by centrifugation, were employed for titration of the agglutinin content. It was found necessary to do agglutination reactions at once with fresh extracts, for on standing, if only a few hours, a fine fibrinous web developed which could not be centrifuged out. This interfered with agglutination reactions however done and such materials were discarded.

It is well known that non-specific flocculation may be encountered when agglutination reactions are done with tissue extracts. It tends to occur chiefly in highly concentrated extracts, is increased in materials that have been incubated or heated and fails to appear, or does so but rarely, when the agglutination reactions are done at room temperature. We have attempted to avoid it by using tissue extracts diluted as much as possible, by the employment of microscopic agglutination methods in those experiments in which tissue extracts had to be used in final dilution as strong as 1 in 32 or 1 in 64 and, lastly, by the employment in all other experiments of the centrifugation method of Gates (8) which requires no heating or incubation. Thus, in our first experiments (see below) mice were injected only once or twice with small amounts of the bacterins and the lymph nodes on the injected and uninjected sides were removed a few days later. In this early work agglutinin formation was inconsiderable and it became necessary to use tissue extracts more concentrated than 1 in 64 in the final dilutions of the agglutination reactions. We employed the microscopic method for these experiments, confirming the results in three of the experiments by the Gates method, using dilutions as strong as 1 in 64. No non-specific flocculation was encountered. In one other experiment shown in Table VII this method was employed with tissue extracts more concentrated than 1 in 64. In all the other experiments in which dilutions of 1 in 64 or more were employed, as well as in those just mentioned, we have used the centrifugation method of Gates (8). This method requires no incubation or heating and is carried out at room temperature. The titrated mixtures of antigen (heat-killed organisms) and diluted tissue extracts or sera were centrifuged for 10 minutes at a speed just sufficient to deposit unagglutinated organisms, about 1400 to 1500 revolutions a minute. Repeated tests of the temperature of the mixtures before and after centrifugation, whirling the thermometers with them, showed

variations of less than 1 degree and never more than 2.5 degrees. A drop of about 0.5° was usual.

All readings were made in a dark room with the tubes held against a black background in such a way that a beam of light entering at the top gave diffuse illumination of the material at the bottom; and all determinations were made under a binocular microscope magnifying about 15 diameters. After inspecting the tubes and noting the character of the sediment as described by Gates (8), they were all gently shaken in the same way while still under the microscope. The flocculi of agglutinated organisms in tubes showing incomplete or partial agglutination could be seen lying like snowflakes upon the homogeneous layer of sediment. On shaking these rose into the supernatant fluid and appeared like snowflakes, while the more closely packed sediment of unagglutinated bacteria remained at the bottom of the tube and only on further shaking rose in a spiral to be distributed through the fluid in an even cloud. If all the sediment rose as large flocculi and these were found floating in a clear fluid the agglutination was called complete. When large flocculi were found in a slightly cloudy fluid, agglutination was deemed incomplete, and it was considered partial if the flocculi were small. The last tube in which small but strong flocculi indubitably remained when examined 10 minutes after shaking, was considered the end-point.

In the experiments in which microscopic agglutination reactions were done only freshly grown, actively motile cultures were used and the fluids to be titrated were measured with 0.1 cc. quantitative Bureau of Standards pipettes. The fluids mixed in porcelain test tablets were used for hanging drop preparations. All end-points were determined 1 hour after mixing. Saline controls were employed in all tests however done. When agglutination occurred in these the tests were discarded.

The antigens used to induce agglutinin formation were, save where specifically mentioned, heat-killed cultures of organisms suspended in saline. As in all but one instance the organisms belonged to the *Salmonella* group, great care was exercised to avoid all R forms, using only the S forms, because of the superiority of the latter organisms as a source of antigen (9–11). To accomplish this end agar subcultures only 16–18 hours old were used which showed the characteristics of S forms. The killed organisms were suspended in 0.2 per cent saline instead of 0.85 or 0.9 per cent, and, in performing the agglutination reactions the sera and tissue extracts were diluted with 0.2 per cent saline to avoid the non-specific agglutination described by Arkwright (9, 10), and which occasionally occurs with employment of physiological saline solutions.

A further effort was made to standardize the suspensions of organisms employed. The killed organisms in each 10 cc. agar slant were removed in ½ cc. of 0.2 per cent saline, and the suspension, diluted five times further with the same solution, was filtered to break up clumps. A suspension of paratyphoid B bacilli was the first to be made up in this way. Later suspensions of the same or different organisms were brought to approximately the same turbidity by diluting until the appearance of

the fluids in capillary tubes of 2 mm. diameter seemed similar to that of the original.

Orienting Experiments

An emulsion of *B. enteritidis*[2] was prepared from agar cultures 16 hours old, heated at 65° for 1 hour. The growths were suspended in ½ cc. 0.2 per cent saline for each slant heated and subcultures were made to make sure the organisms were dead. When this had been proven by lack of growth the suspension was further diluted with 0.2 per cent saline to a turbidity similar to that of the paratyphoid bacterin.

TABLE I

Agglutinins for B. enteritidis in Cervical Lymph Nodes and Sera of Mice Injected in Both Ears with Killed Cultures on 2 Successive Days

7 days following the last injection	Dilutions of the gland substance and sera	Lymph nodes	Sera
Group I. Microscopic method	1 in 32	++	+
	64	+	0
	128	0	0
Group II. Centrifugation method	1 in 32	Not done	+
	64	++	0
	128	+	0
	256	0	0
Group III. Centrifugation method	1 in 16	Not done	+
	32	Not done	0
	64	++	0
	128	0	0

++ = incomplete agglutination.
+ = partial agglutination.

A group of 30 mice of about 25 gm. body weight was injected intradermally in both ears with about 0.02 cc. of the *enteritidis* bacterin. 7 days after the last injection the enlarged lymph nodes of the neck were removed from the etherized animals and they were bled for serum. In every instance the nodes were inflamed and much enlarged. The lymph glands from the individuals of this group were ground together and the sera pooled, thus obtaining one node extract and one serum specimen. These were titrated for agglutinins, using the microscopic method as already described. The experiment was later twice repeated and the agglutination reactions done by the centrifugation method of Gates (8) for con-

[2] Culture obtained from Dr. Leslie T. Webster, The Rockefeller Institute for Medical Research.

firmation. Table I shows the dilutions of the gland substance and sera at which strongly positive agglutination was found.

In these experiments agglutinins were found in both nodes and sera. They were much stronger in the former.

In a second experiment an emulsion of paratyphoid B bacilli was prepared from agar cultures 18 hours old, and killed by heating to 65°C. for 1 hour in a water bath. After subculture to assure sterility, it was diluted with 0.2 per cent saline as in the case of *B. enteritidis* bacterin. On successive days two groups of 20 mice each were given intradermal injections in both ears of 0.03 cc. of the bacterin, and 5 days

TABLE II

Agglutinins for B. paratyphosus B in Cervical Lymph Nodes and Sera of Mice Injected on 2 Successive Days in Both Ears with Killed Cultures

Interval of 5 days after last injection	Dilutions of the gland substance and sera	Lymph nodes	Sera
I. Microscopic method	1 in 32	++	±
	64	+	0
	128	0	0
II. Microscopic method	1 in 32	++	+
	64	+	±
	128	0	0

++ = incomplete agglutination.
+ = partial agglutination.
± = weakly or dubiously positive agglutination.

after the last injection, that is to say 2 days sooner than in the previous experiment, lymph node extracts and sera were procured. Agglutination reactions were done with the microscopic technique.

As Table II shows, agglutinins were again stronger in the node extract than in the sera but they were present in both.

Antigens were next injected intradermally in one ear of each of a series of animals, and the time of appearance of antibodies was roughly determined in the cervical nodes of the different sides, in the serum, in the tissues of the injected and untouched ears, and in certain other organs, notably the liver and spleen.

A large number of mice of about 30 gm. body weight were injected intradermally in the right ear only on 2 successive days with approximately 0.03 cc. of the paratyphoid bacterin used in the previous experiment. On the 1st, 2nd, 3rd, 5th, 7th, 8th, 10th and 12th days after the last injection, random groups of ten animals were anesthetized with luminal, bled for serum and the cervical lymph nodes on both the injected and control sides were removed. In every instance the nodes on the injected side were greatly enlarged and at times hemorrhagic, while those draining the uninjected ears appeared normal. Serum and node extracts were titrated for agglutinins, using the microscopic technique. The results are summarized in Table III.

As the table shows, no antibody was found until the 7th day following the last injection. It then appeared simultaneously in the lymph nodes of the injected side and in the serum, but in much higher concentration in the former. The lymph nodes of the uninjected side contained no demonstrable agglutinins. As the experiment progressed the antibody concentration in nodes and serum increased, with always a little more in the former. On the 10th day agglutinins were found in the extract of ear tissue on the injected side but not until the 12th day did they appear in the nodes of the uninjected side. At this time, too, they first appeared in the spleen, a fact not shown in the table. They were not found in the liver nor in the ear tissue on the uninjected side.

No attempt was made to establish the earliest moment at which agglutinins could be demonstrated, since this would largely depend on the dosage and nature of the antigen.

Objections to the Experiment.—This experiment served to demonstrate the early appearance of agglutinins in the regional lymph nodes and serum after intradermal injection of antigens, and its later presence in the ear tissue on the injected side and in the spleen. But nothing can be concluded from it as to the site of antibody formation. The lymph nodes on the right side were found inflamed and hypertrophic, those on the left normal. The former contained a slightly higher concentration of agglutinins than the serum, and until the 12th day none was found in the lymph glands of the left side, yet it is conceivable that the inflamed nodes took up from the blood antibody which had been formed elsewhere, while the normal lymph nodes failed to do so. The injections led to inflammation in the right

TABLE III

Agglutinins in Cervical Lymph Nodes, Sera and Ear Tissue of Mice Receiving on 2 Successive Days Intradermal Injections of B. paratyphosus B Bacterin in the Right Ear Only

Days after last injection	Dilutions of the tissue substances and of sera	Injected side Right node	Control side Left node	Serum	Injected ear Right	Control ear Left
1	1 in 30	0	0	0	0	0
	60	0	0	0	0	0
	120	0	0	0	0	0
	240	0	0	0	0	0
	480	0	0	0	0	0
2	1 in 30	0	0	0	0	0
	60	0	0	0	0	0
	120	0	0	0	0	0
	240	0	0	0	0	0
	480	0	0	0	0	0
3	1 in 30	0	0	0	0	0
	60	0	0	0	0	0
	120	0	0	0	0	0
	240	0	0	0	0	0
	480	0	0	0	0	0
5	1 in 30	0	0	0	0	0
	60	0	0	0	0	0
	120	0	0	0	0	0
	240	0	0	0	0	0
	480	0	0	0	0	0
7	1 in 30	++	0	±	0	0
	60	+	0	±	0	0
	120	+	0	±	0	0
	240	0	0	0	0	0
	480	0	0	0	0	0

+++ = complete agglutination.
++ = incomplete agglutination.
+ = partial agglutination.
± = weakly or dubiously positive agglutination.

TABLE III—*Concluded*

Days after last injection	Dilutions of the tissue substances and of sera	Injected side Right node	Control side Left node	Serum	Injected ear Right	Control ear Left
8	1 in 30	++	0	++	0	0
	60	++	0	+	0	0
	120	+	0	±	0	0
	240	0	0	0	0	0
	480	0	0	0	0	0
10	1 in 30	++	0	++	+	0
	60	++	0	+	±	0
	120	+	0	±	±	0
	240	±	0	0	0	0
	480	0	0	0	0	0
12	1 in 30	+++	++	+++	+	0
	60	+++	+	+++	±	0
	120	+++	+	+++	±	0
	240	+++	±	+	0	0
	480	++	0	±	0	0

ears with accompanying dilatation of blood vessels in the inflamed areas, and it is well known that dilated vessels are more permeable than normal. It follows that agglutinins present in the blood might have escaped into the interstitial tissues of the ear, there to be drained by the lymphatics to the regional node and held in high concentration. Earlier workers, seeking to determine the sites of antibody formation have injected antigen intravenously and then attempted to demonstrate antibodies in this or that organ extract prior to its appearance in the blood. Their results, like ours thus far recorded, though suggestive of the site of origin of antibodies, fail to prove it for reasons like those just given.

Experiments Demonstrating the Origin of Agglutinins within Lymph Nodes

Three types of experiment were devised to control the possibilities just discussed.

In the first type, inflammation was induced in the cervical nodes of both sides and in both ears by the injection of paratyphoid bacterin

on one side and diphtheria toxin on the other. The latter was utilized to produce local inflammation without introducing an agglutinin-forming antigen. In these experiments the ears and nodes on both sides became inflamed and swollen but not quite to the same degree, the nodes on the side injected with paratyphoid bacterin becoming slightly larger. The possible effects of the difference will be considered below.

In the experiment yielding the results recorded in Table IV, about 0.03 cc. of the paratyphoid B bacterin was injected intradermally on 2 successive days into the right ears of 20 mice. Schick test toxin 0.03 cc. was injected intradermally at the same time into the left ears. After 7 days more the animals were bled under ether anesthesia and the nodes on the right and left sides were extracted and titrated for agglutinin content by the microscopic method. Sections of the nodes from both sides appeared similar under the microscope.

As Table IV shows, paratyphoid agglutinins were present in the extract from the right nodes, that is to say, on the side injected with killed paratyphoid organisms. Agglutination was strongly positive at a dilution of 1 to 120 and faintly positive at 1 to 240. Much less agglutinin was present in the serum, tests being positive at 1 to 60 and negative at 1 to 120. No agglutinins were demonstrable in the extract of the nodes on the side injected with diphtheria toxin. The ears on this side were markedly inflamed but slightly less so than on the opposite side.

If, in the earlier experiments, agglutinins formed elsewhere in the body were taken out of the blood by the inflamed nodes on the injected side or had seeped through the permeable blood vessels of the inflamed ear to be drained to the lymph nodes and accumulate there, surely some should have been found in the present experiment on the side on which diphtheria toxin was injected. This was not the case and so the findings indicate that the antibodies present in the right lymph nodes had been formed there.

The experiment still left much to be desired. The nature of the antigens differed, one an emulsion of killed bacilli, the other a cell-free fluid toxin, and it seemed possible that the results might in some way depend upon this difference. For example, in the ear injected with the emulsion of bacilli, many of the organisms no doubt remained *in situ* for a time. Lymph drainage from such an area might con-

ceivably carry antibodies formed in the ear to the regional nodes, there to be concentrated. To control this factor, a second type of experiment was devised.

TABLE IV

Agglutinins for B. paratyphosus B in the Sera and in Extracts of the Right and Left Cervical Nodes after Injections into Both Ears

	Dilutions of the gland substance and serum	Right node extract Right ear injected with paratyphoid bacterin	Left node extract Left ear injected with Schick test toxin	Serum
2 intradermal injections on successive days; interval of 7 days	1 in 60	++	0	+
	120	+	0	0
	240	±	0	0
	480	0	0	0

++ = incomplete agglutination.
+ = partial agglutination.
± = weakly or dubiously positive agglutination.

TABLE V

Agglutinins for B. paratyphosus B in the Serum and in Extracts of Right and Left Cervical Nodes after Injections Into Both Ears

1 intradermal injection only, 2 hrs. later both ears removed; interval of 9 days	Dilutions of the gland substance and serum	Right node extract Right ear injected with paratyphoid bacterin	Left node extract Left ear injected with Schick test toxin	Serum
Exp. I. Microscopic method	1 in 60	+	0	±
	120	±	0	0
	240	0	0	0
Exp. II. Centrifugation method	1 in 64	+	0	+
	128	+	0	0
	256	0	0	0

+ = partial agglutination.
± = weakly or dubiously positive agglutination.

Twenty mice were injected intradermally in the right ear only and but once, with 0.03 cc. of the paratyphoid B bacterin. Diphtheria toxin was injected in the left ears. 2 hours later, under ether anesthesia, both ears were amputated. 9 days later the nodes on both sides were removed and serum obtained. In this experiment agglutination reactions were done by the microscopic method. Later it was repeated employing the centrifugation method. Table V gives the results

of the titrations. The nodes on both sides were much enlarged and inflamed, on the right side slightly more than on the left. Sections of both nodes were similar.

No paratyphoid agglutinin was found in the nodes of the left side, injected with Schick toxin. The extract from the nodes on the other side was positive at a dilution of 1 to 128 in one experiment and weakly so at 1 to 120 in the other. The tests of serum were negative in those dilutions though positive or weakly so at 1 to 64 or 1 to 60 respectively.

This experiment strongly suggests the formation of antibody within lymph nodes, for the nodes on the left side yielded no demonstrable agglutinin; antibody had not passed to them from the blood in spite of the inflammation. The agglutinin present in the nodes on the side injected with paratyphoid bacterin did not derive from the seepage of antibodies from the blood into the interstitial tissue of the ear with subsequent drainage to the node by way of the lymphatics, for the ears had been removed. Complete proof of the formation of antibody by the lymph nodes was not obtained because of the slight difference in the degree of inflammation on the two sides which might be held accountable for the result.

In two other groups of mice both ears were injected on two successive days with the paratyphoid bacterin. 2 days later, at a time when no agglutinins could be demonstrated in either blood or nodes, the left ears were amputated. This was done to prevent the possibility of a seepage of agglutinin in the blood through the walls of the dilated blood vessels of the inflamed ear and its drainage to the node on that side. As a control the right ears were left intact. 6 and 7 days after the last injection, respectively, the two groups of animals were killed and node extracts and sera obtained. Agglutination reactions were done by the microscopic method. As Table VI shows, the titre of agglutinin in the nodes of the two sides was equal, ruling out once again the possibility that the agglutinin in the node was brought to it from the ear. Further, microscopic studies of the nodes from both sides showed no differences.

Proof of the Formation of Agglutinins within Lymph Nodes

A third type of experiment was devised to show finally whether or not agglutinin formation takes place within lymph nodes. Different

antigens of a similar nature were employed; that is to say, suspensions of various killed organisms which would call forth agglutinin formation, and at the same time induce approximately the same degree of inflammation in the injected ears and in the regional lymph nodes.

One bacterial suspension was injected intradermally into the right ears and the other into the left ears. Several days later the titre of both specific agglutinins was determined in the sera and lymph node

TABLE VI

Agglutinins for B. paratyphosus B in the Sera and in Extracts of Right and Left Cervical Nodes and in the Sera of Mice Injected as Described in the Text

	Dilutions of the gland substance and sera	Both ears injected with killed cultures of *B. paratyphosus* B		Sera
		Right node extract	Left node extract	
2 intradermal injections in both ears on successive days; left ear removed 2 days later; interval of 6 days	1 in 60	++	++	++
	120	±	+	+
	240	±	±	±
	480	0	0	0
Interval of 7 days	1 in 60	++	++	++
	120	++	++	++
	240	+	+	+
	480	±	±	±

++ = incomplete agglutination.
+ = partial agglutination.
± = weakly or dubiously positive agglutination.

extracts from both sides as will be described below. Organisms were used which gave no cross-agglutination.

In a preliminary experiment five series of ten mice each were injected three times, on successive days, intradermally in both ears, with about 0.02 cc. of heat-killed emulsions of five different organisms, *B. typhosus*, *B. paratyphosus* B, *B. enteritidis*, *B. coli* and *B. prodigiosus*, one organism only in each series of mice. The bacterins were prepared in the usual manner from 16–18 hour agar slants of the organisms, killed by heating and diluted until each appeared as turbid as the suspensions of organisms used in the preceding experiments. Every 2nd day, following the injections, two of each series were killed and the lymph nodes removed for comparison. In all a pronounced inflammatory swelling of the lymph

nodes occurred, *B. prodigiosus* producing marked lymphangitis and enlargement. This was to be expected from the recent findings of Rosahn (12), who reported definite pathological changes in mice injected intravenously with *B. prodigiosus*. Two other groups, each of 20 mice, were then injected with *B. enteritidis* and *B. prodigiosus* respectively. After 2 weeks the sera of these mice were tested for cross-agglutination and none found. Sections of the lymph nodes inflamed by the two organisms showed no differences when studied under the microscope. Since similar lymph node swellings were obtained and separately distinguishable specific agglutinins were present in the node extracts, these two organisms were used for the following experiments.

Forty-five mice, approximately 30 gm. in weight, were given three intradermal injections of the two bacterins on successive days. In the right ears 0.02 cc. of *B. enteritidis* suspension was injected and in the left the same amount of killed *B. prodigiosus*. On the 8th day after the last injection the lymph nodes and sera were taken. The nodes of the same side were pooled in two lots, one of which was ground and extracted with 0.2 per cent saline solution, the other with the mixture of equal parts of the saline solution and glycerine. To half the pooled serum glycerine was added in amount corresponding to that present in the node extracts.

Determinations of agglutinin concentrations were made upon the fresh material, using the centrifugation method of Gates (8) (Experiment 1 *b*, Table VII), and also the microscopic method (Experiment 1 *a*, Table VII). As the end-points of the titrations showed no significant difference between the glycerine-saline and the plain saline solution extracts, only the results with the latter are given in the table.

The highest concentration of *B. enteritidis* agglutinin was found in the extracts of the lymph nodes from the side injected with that antigen, two to four times as much as in the serum. The serum in turn contained more agglutinin for *B. enteritidis* than did the extract of the nodes from the other side. This despite the fact that the nodes were equally inflamed, as judged by their size and appearance in the gross and in microscopic sections. In a corresponding manner the extract of lymph nodes from the side injected with killed *B. prodigiosus* contained the highest concentration of *B. prodigiosus* agglutinin with much less in the serum and least in the lymph node extract from the side injected with *B. enteritidis*.

It is noteworthy that the concentration of each of the agglutinins was greatest in the lymph nodes on the side injected with the corresponding antigen and least in the lymph nodes on the opposite side. The concentration in the serum stood midway between the two. Had agglutinins been formed elsewhere than in the nodes, this distribution could not have occurred.

The experiment was twice repeated with similar results (Experiments 2 and 3, Table VII), using in this and the subsequent experiments the centrifugation method for agglutinin determinations. In another trial a group of mice were four times injected in the same way and examined after a 10 day interval (Experiment 4, Table VII).

TABLE VII

Agglutinins for B. enteritidis and B. prodigiosus in the Sera and in Extracts of the Right and Left Nodes of Mice Injected in the Right Ears with B. enteritidis and in the Left with B. prodigiosus

Procedure	Experiment No.	Titre of *B. enteritidis* agglutinins			Titre of *B. prodigiosus* agglutinins		
		In extract from nodes on side injected with *B. enteritidis*	In sera	In extract from nodes on side injected with *B. prodigiosus*	In extract from nodes on side injected with *B. enteritidis*	In sera	In extract from nodes on side injected with *B. prodigiosus*
3 intradermal injections on successive days, 8 day interval after last injection	1*a*	1 in 256	1 in 128	1 in 64	1 in 128	1 in 512	1 in 1024
	1*b*	256	64	1 in 32	1 in. 64	256	512
	II	256	64	64	64	256	256
	III	256	128	32	64	512	1024
4 injections on 4 successive days, 10 day interval after last injection	IV	1 in 512	1 in 64	1 in 32	1 in 256	1 in 256	1 in 512
7 injections in 2 wks., 21 day interval after the first injection	V	1 in 2048	1 in 1024	1 in 512	1 in 1024	1 in 1024	1 in 4096

In a final experiment of the same type, mice were highly immunized by six injections of antigen given within a period of 9 days. 5 days later they were injected once more and a week thereafter (Experiment 5, Table VII), *enteritidis* agglutinins were most concentrated in the extract from the lymph nodes on the side injected with that antigen. The serum contained a high titre of the same agglutinin, almost as high as did the lymph node extract. But the material from

the nodes on the other side showed the least agglutinin for this organism. Almost similar findings resulted from the determination of agglutinins to *B. prodigiosus*. The highest titre occurred in the node extract from the side injected with killed *B. prodigiosus*. It was much less in the serum and in the node extract from the side injected with *B. enteritidis*.

It is to be noted in this connection that in all but one of these experiments (1 *a*, Table VII) the agglutination reactions were done by the centrifugation method, using node extracts in these experiments as strong as 1 in 32 or 1 in 64. As the figures enclosed in broken lines, in columns 5 and 6 show, positive agglutination occurred at this concentration for *B. prodigiosus* in the extract of nodes from the side injected with *B. enteritidis* (column 6), and *vice versa* in column 5. At such concentrations the effect obtained may have been due to non-specific flocculation. However in Experiments IV and V in this table, in which the animals received more injections of antigen and as result were highly immunized, we still found the same effect even at high dilutions. We conclude therefore that our findings are due to true agglutination in all the experiments shown in the table. Should the effect shown in columns 5 and 6 have been due to non-specific flocculation in any of the experiments it would serve as still more definite evidence that agglutinins for a bacterium are formed only in the nodes of the side injected with that organism.

Variations in the Concentration of Agglutinins in Lymph Nodes and Serum

The findings show clearly a formation of agglutinins within lymph nodes. And in the course of the experiments, other evidence was obtained pointing to the phenomenon. The titre of antibody in the lymph nodes and sera varied greatly with the conditions of the experiment. When antigen was injected only once or twice and the concentration of antibody in serum and lymph node extract sought shortly thereafter, far more agglutinin was found in the latter than in the former. But when repeated injections of antigen were made and the interval between the time of first injection and examination was delayed, for example for 12–21 days, the titre of antibody both in lymph node extract and serum was greatly increased but the concen-

tration of agglutinins rose more rapidly in the serum, eventually in one instance exceeding that of the nodes.

Table VIII has been prepared to show this point, using for data experiments which were done with a single antigen, *B. enteritidis*, and in which the agglutination tests were done in the same way, by the Gates method. The findings as just discussed above constitute further evidence for the formation of antibody in the lymph nodes under the conditions of the experiments and its subsequent distribution to

TABLE VIII

Changing Ratio of Agglutinins for B. enteritidis in Nodes and Sera

No. of intradermal injections	Interval from first to last injection	Interval after last injection	Positive agglutination from lymph node substance diluted	Positive agglutination in sera diluted
2 In both ears	Successive days	7 days	1 in 128	1 in 32
			64	32
			64	16
			128	32
3–4 In one ear only	Successive days	8 days	1 in 256	1 in 64
			512	64
5 In both ears	14 days	6 days	1 in 1024	1 in 1024
			512	512
			1024	1024
5 In both ears	11 days	7 days	1 in 1024	1 in 1024
5 In both ears	15 days	6 days	1 in 2048	1 in 1024
			1024	1024
			1024	2048

the serum in ever increasing amounts. No doubt were one to compare the concentration of agglutinin in lymph node extract and serum at still longer intervals, one might find little or no agglutinin in the lymph nodes and much in the blood. From these and from other findings mentioned above, it is plain that one cannot determine the concentration of antibody in blood or organs at one time only and draw conclusions about the site of antibody origin. Early in the process of immunization antibodies may exist in higher concentration

in the lymph nodes than in the blood but late in the process the findings might be reversed.

DISCUSSION

The experiments just described demonstrate the formation of agglutinins by lymph nodes. The finding throws some light upon the phenomena of defense to infection entirely by way of the skin and mucous membranes. In earlier papers from this laboratory (1) we have shown that every scratch, puncture or incision of skin ruptures the minute lymphatic capillaries and that, when ruptured or incised, they remain open (2, 5) for many hours, affording an opportunity for infection. Somewhere along the lymphatic channels body defenses must be active. When vital dyes are placed in superficial cuts (1, 2) they are drained by the lymphatics to the regional nodes. Our present work shows that in the lymph nodes there exists a strong humoral defense mechanism appropriately situated upon the route of the invading infection. Earlier work of others has shown that lymph nodes sieve out large numbers of bacteria injected intravenously or subcutaneously (13–15) and hold them *in situ*. It seems only natural that antibodies should be formed in very high concentration in the organ in which antigen is itself held.

Much has been written of the mechanism by which so called local injections of vaccine or other antigens confer general immunity. Our earlier paper (1) showed that intradermal injections are largely intralymphatic and substances so introduced are rapidly carried by the lymphatics to the regional lymph nodes even in the resting arm. The same must be true when antigen is clinically injected intradermally in a patient's arm to produce a "local" reaction. In view of the findings now reported, one must suppose that some part of the immunity conferred by the procedure is developed through the activities of the lymph glands.

From certain of our findings it seems probable that an intradermal injection need not be wholly intralymphatic in order to incite a formation of antibodies in the lymph nodes. That is to say, lymphatic capillaries need not be broken in large numbers to cause transport of the antigen to the nodes; for apparently lymphatic absorption occurs whether the channels are injected or not. For example, in repeatedly

injecting the ears of our mice, it was noticed that the texture of the skin varied greatly. The thin-skinned ears became badly damaged after a few inoculations and one could no longer effect true intralymphatic injections, yet, to judge by the reaction of the lymph nodes of these animals at autopsy, much of the absorbed material must have been carried to them by the lymphatics, for the nodes were inflamed and enlarged equally with those of tough-skinned animals, in which the injections were definitely intralymphatic.

That local injections produce speedy, remote reactions by vascular absorption is of course a truism as shown by the action of injected drugs; but the rapid lymphatic distribution of antigen is perhaps not so generally recognized. For example, Oshikawa, cited by Hoder (16), injected killed *proteus* bacilli into the ear of a rabbit and 10 minutes afterwards cut off the ear. He sought and found agglutinins in the blood later. Reitler, also cited by Hoder (16), found agglutinins in blood, to *B. coli* and *B. mesentericus*, when the ear was amputated 3 seconds after a subcutaneous injection. These authors attribute the escape of antigen from the ear to rapid absorption by the blood. From our findings it seems probable that antigen was directly injected into the lymphatics in such experiments and distributed to regional lymph nodes where antibodies were formed. The same result might readily follow intracutaneous immunization as done by Tuft (17). Absorption of some of the residue of an intradermal injection as well as part of the originally injected fluid seems to be by way of the lymphatics.

In the past many authors have sought to determine the place of antibody formation by local or intravenous injection of antigens followed by the extraction of various organs for antibody. Depending upon the site of antigen injection antibodies have been found, now in this organ now in that, in higher concentration than in blood or occasionally before its appearance therein.

As early as 1898, Pfeiffer and Marx (18, 19) titrated the bacteriolysin content of various organs and of the blood of rabbits intravenously injected with killed cholera spirilla. They reported the antibody titre in the spleen, bone marrow, lymph glands, lungs and blood. Bacteriolysin was at times found in higher concentration in the spleen than in the blood and in two or three instances appeared first in the spleen on the 2nd day after injection. Somewhat similar findings were

reported by A. Wasserman (20), using typhoid organisms as antigen, and by M. Wasserman employing pneumococci (21).

Following the injection of killed hog cholera bacilli into the mesenteric vein of rabbits, Jones (22) found a higher titre of agglutinin in the liver than in blood or other organs. He showed further that in animals highly immunized the agglutinin titre was highest in serum, but in animals killed early in the process agglutinin was most abundant in the liver. In 1923 (23) Theobald Smith, Orcutt and Little showed that inoculations of *B. abortus* in the various quarters of cows produced agglutinins in the milk and the antibodies were found in highest concentration in the milk coming from the infected quarter. Local antibody formation in the skin has been stressed by Fernbach and Hässler (24), Cannon and Sullivan (25), Cannon and Pacheco (26) and others, and in mucous membranes by Walsh, Sullivan and Cannon (27). Seegal and Seegal (28) showed that the injection of typhoid vaccine into the anterior chamber of the rabbit's eye resulted in a concentration of agglutinins in certain tissues of the eye. Antibodies have been found in varying concentrations in blood, thoracic duct lymph, cervical lymph, cerebrospinal fluid and aqueous humor (29–31). Since the relative concentrations of antibody are similar in actively and passively immunized animals, the findings have yielded no clue as to their place of origin.

Much, too, has been written upon the function of the reticulo-endothelial system in the process of immunization. A discussion of this subject would take us too far afield; but it is conceivable that the cells of this system which are present in the lymph nodes or elsewhere may be responsible for agglutinin formation.

The evidence cited, for local formation of antibodies, though highly suggestive, fails to prove their formation within the tissues investigated: for the mere finding of antibody in high concentration within an organ does not prove its formation therein. The blood vessels in inflamed regions are more permeable than normal and might readily allow antibody to pass from the blood into the tissues with accumulation there. This objection which applies to most of the earlier work of others we have raised against our own experiments in the beginning of this paper, and various devices were employed to overcome it in the later experiments.

That lymph nodes take part in immunity reactions has long been assumed; but almost every tissue in the body and every organ has been considered as the place of antibody formation.

Lymph nodes have long been known to sieve out bacteria (14, 15) and hold them, and definite inflammatory reaction occurs in the nodes when toxins and bacteria are injected intradermally or subcutaneously. As early as 1890 Oertel (32) noticed that lymph glands were affected in diphtheria, that edema of the glands

occurred and histologically the germinal centers became involved. In 1891 Welch and Flexner (33) inoculated diphtheria bacilli subcutaneously into kittens, rabbits and guinea pigs and described marked changes in the regional glands draining the site of injection. Lesser changes occurred in the lymph glands in other parts of the body. Barbacci (34) confirmed this 5 years later and clinical observations by Bulloch (35) report the finding of enlarged lymph glands in patients with diphtheria.

Councilman (36) called attention to the possible relationship of collections of lymphoid cells to the production of immunity, and, in the following years the significance of these collections of lymphocytes has been much investigated. To judge from the careful studies of Murphy and his collaborators (37) there would appear to be some relation between the lymphocyte and immunity to tumors. Further, Murphy and Sturm (38) showed that rabbits subjected to dry heat showed increased activity of the lymphoid tissue and developed a higher titre of agglutinins to Pneumococcus Type I and precipitins to horse serum than did untreated control animals. Other rabbits formed far less of these antibodies following suitable X-ray treatment which reduced the amount of lymphoid tissue.

More recently Takahashi (39) has reported upon the subject of antibody formation in lymph glands. In rabbits immunized against human red cells by intravenous injections over a period of 3 weeks, agglutinins were found in blood serum and in peripheral lymph flowing to and from lymph nodes. This finding is taken as evidence for the formation of agglutinins by the glands themselves. In immune animals, however, agglutinins may be found in practically all the body fluids (29–31) if present in the blood, and the very slight differences found by this worker in the agglutinin content of minute amounts of inflowing and outflowing lymph may easily be accounted for by errors in the method or by changes in the water content of lymph passing through the gland.

For many years, too, morphological evidence has been accumulating to show that the lymphatic system participates in the processes of immunization. Matko (40) described marked changes in the lymph glands within 3 days following vaccination with typhoid "vaccine." Hellman and his coworkers (41, 42) showed that there occurred an increase in the total lymphatic tissues of rabbits of different ages during the process of immunization to paratyphoid bacilli. Antigen was injected intravenously and the spleens showed great increase in size thereafter, especially in their secondary nodules. The lymphatic tissue in the intestine and tonsils increased in amount. From his histological findings Hellman believed that the secondary nodules in the spleen and lymph glands are centers reactive against bacteria and other toxic agents entering the organs. In 1929, Ehrich (43, 44) described the changes in lymph glands after subcutaneous and intravenous injection of killed staphylococci. Enormous enlargement of the cortex of the lymph glands developed, hand in hand with changes in the number of circulating lymphocytes.

Finally the formation of antibodies has been sought in tissue culture by Carrel and Ingebrigtsen (45) who demonstrated the formation of hemolysins by a mixture of bone marrow and lymph glands. Meyer and Loewenthal (46) were unable to produce agglutinins in tissue cultures of spleen, lymph nodes or mesenteric milk

spots when antigen was directly added. However, when living animals were first injected with antigen and the organs removed later and cultured *in vivo* agglutinins were demonstrated.

One further point deserves emphasis. The experiments described in this work were planned to demonstrate whether or not agglutinins are formed within lymph nodes; the findings throw no light on the subject of antibody formation elsewhere in the body. There seems to be no good reason to consider the lymph nodes as the sole site of antibody origin.

SUMMARY

Agglutinins are formed within the draining lymph nodes of mice, following intradermal injections of killed cultures of microorganisms.

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