THE ABSENCE OF ANTIBODY IN THE MACROPHAGES DURING MAXIMUM ANTIBODY FORMATION*

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In previous experiments (1) it was found that following the injection of various antigens into the hind feet of rabbits antibody first appeared in two places, namely in the popliteal lymph node, the only node regional to the site of injection, and in the lymph contained in the efferent lymph vessel of this node. The highest titers in node and lymph were reached after 6 days. In all experiments the titer was higher in the efferent than the afferent lymph; in some cases the concentration was 100 times that found in the afferent lymph. The appearance of antibody in the lymph node was preceded and accompanied by greatly increased lymphocytopoiesis: numerous lymphoblasts made their appearance; there was marked mitotic activity; the node became rapidly filled with a diffuse lymphoid tissue. Simultaneously, there was a sharp rise in the output of lymphocytes into the efferent lymph.

When, at the time of greatest antibody production in the lymph node, lymph was collected from the efferent lymph vessel, and the concentration of antibody within the lymph cells was compared with that of the lymph plasma (2), it was discovered that the lymph cells in many instances contained from 8 to 16 times as much antibody as the lymph plasma. As 99 per cent of the cells were found to be lymphocytes, and *in vitro* and *in vivo* experiments failed to show absorption of antibody by these cells, it was concluded that the lymphocyte was instrumental in the formation of antibody.

When we spoke of lymphocytes, we meant the usual small lymphocytes and their predecessors. The morphologic appearance of these cells is well illustrated in Fig. 12 of a previous publication (3). Whether or not other lymphoid cells, such as the plasma cells, also produce antibodies or other globulins has not been determined. The frequent observation of hyper-gamma-globulinemia and hyper-beta-globulinemia in multiple myeloma suggests that this may be the case. So far, however, the lymphocyte is the only cell in which antibodies have been demonstrated.

Recently, White and his associates (4–7) have demonstrated the presence of large quantities of labeled and normal gamma globulin, and possibly also beta globulin, in extracts of lymphoid cells derived from minced pools of lymph nodes and thymus

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glands of mice. Though these cells, as recently stated (8), probably consisted mostly of lymphocytes, it cannot be doubted that considerable numbers of plasma cells were present also, as these are a prominent feature in the lymph nodes of small laboratory animals. It is not apparent, therefore, which cells were the sources of the globulins which they recovered. The contention of White and his associates that antibodies were contained in lymphocytes may be regarded as valid, however, in the light of our work where an almost pure strain of lymphocytes was used (2).

Although a lymphocytic theory of antibody formation had thus been established, it had not been ruled out that other cells that are found in inflammation might also be instrumental in antibody synthesis. Nor had it been shown whether the lymphocyte produced globulins other than the antibodies studied.

The present communication deals with the rôle of the macrophage in antibody formation. It will be shown that the macrophages do not contain significant quantities of agglutinin during the formation of this antibody. This finding together with the previously described observations on the lymphocyte seem to show that at least the synthesis of the antibodies studied thus far is not a function of the macrophage.

Methods

As in our previous experiments (1, 2), male Chinchilla rabbits weighing about 2000 gm. were used whenever available. With the exception of a small series of animals to which typhoid vaccine was given, all experiments were performed with dysentery antigen. The typhoid vaccine was prepared as previously described (1, 2); for dysentery vaccine, we used alcohol-killed cells of *Shigella paradysenteriae* Flexner Z911A.[1]

Cell extracts for antibody determination were prepared as described previously (2). As soon after collection as feasible the various fluids containing the cells were centrifuged at 1600 R.P.M. for 10 minutes. After the supernatant fluid had been drawn off as completely as possible, the cells were washed twice with several volumes of physiologic saline solution. The sediment was then placed alternately in an ice bath of approximately −70° C. and in a water bath of +37° C. After 3 cycles of freezing and thawing the material was spun at 5000 R.P.M. for 20 minutes to remove the cellular debris. The supernatant fluid of this material served as cell extract.

In the typhoid experiments, agglutination was tested as described previously (2). In the dysentery experiments, the tests were performed as follows:—

Serial dilutions in steps of two were made of the material to be examined for antibody concentration. To 0.4 cc. quantities of such dilutions was added 0.2 cc. of a suspension of the *Shigella*. The organisms had been grown on tryptose agar slants after 16 hours of incubation at 37° C., exposed to 70 per cent ethyl alcohol for 3 hours, washed free of the alcohol, and finally suspended in a concentration of 0.5 mg. dry weight per cc. The tubes were then shaken, incubated for 1 hour at 37° C., and stored overnight in the refrigerator. Readings were made by the pattern of the sedimented bacterial suspension as well as by agglutination.

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[1] This strain was obtained from the Department of Bacteriology of the School of Medicine of the University of Pennsylvania; it was received originally from the Puerto Rican Department of Laboratories.

RESULTS

1. The Relative Antibody Titers in the Foot Pad, the Popliteal Lymph Node, and the Blood Serum after Subcutaneous Injection of Dysentery Vaccine in Saline, and in Saline-in-Paraffin-Oil Emulsion

In our search for a method to study the rôle of the macrophage in antibody synthesis, two avenues of approach presented themselves. The first grew out of a recent investigation of the augmenting action of mineral oil on antibody production, and the various tissue reactions that occurred at the site of injection in the regional lymph node and elsewhere (9). It was found that antigen in oil produced a more intense and longer lasting antibody response than antigen in saline. At the site of injection, the reaction to antigen in saline was chiefly one of catarrhal inflammation, while that to antigen in oil was one of suppuration followed by the production of large granulomata consisting almost purely of macrophages. In the regional lymph node, we first found a diffuse lymphoid hyperplasia with numerous dividing lymphoblasts, while later, when the antibody titer had reached its peak, large secondary nodules made their appearance; these reactions were more intense and longer lasting in the animals injected with antigen in oil. There was no correlation between antibody formation and the local changes at the site of injection. However, the changes in the lymph nodes closely paralleled the antibody response, both in intensity and in duration. These findings seemed to indicate that the antibody arose in the regional lymph node, and not in the cells at the site of injection.

Extending these experiments, we now determined the antibody content of the tissue at the site of injection, the regional lymph node, and the blood serum. Dysentery organisms suspended in saline, or in saline-in-paraffin-oil emulsion, stabilized with Falba, a lanolin-like substance, prepared according to a procedure described elsewhere (9), were injected into the foot pads, 0.25 mg. into the right, 1.25 mg. into the left. The animals were sacrificed at various intervals up to 14 days after injection.

The results of these experiments are given in Table I. The titers in the blood serum closely resembled those recorded previously (9): in the antigen-in-oil experiments they reached their peak later and stayed there longer than in the antigen-in-saline experiments. Antibody production in the lymph nodes like that after the injection of typhoid vaccine in saline (1), reached its peak on the 5th and 6th days; after injection of antigen in oil this production was not only of greater intensity, but it reached its peak later and it lasted longer. On the other hand, the titers at the site of injection remained insignificant in both series of experiments; like the titers in the blood serum, they were slightly higher in the antigen-in-oil experiments.

Considering the origin of the antibody, it appeared that the titers at the

site of injection were of a magnitude that one would expect if the antibody arrived there secondarily through concentration (fixation); also, no correlation was found between titers and tissue changes at the site of injection. On the other hand, good correlation existed between the changes in the regional lymph

TABLE I

Comparison of Antibody Titers in Foot Pads, Lymph Nodes, and Serum, after Injection of Dysentery Vaccine in Saline, and in Saline-in-Paraffin-Oil Emulsion

Antigen	Time after injection of antigen	Rabbit No.	Titer at site of injection		Titer of lymph node extract		Titer of blood serum
			Rt.*	Lt.*	Rt.*	Lt.*	
	days						
	3	3-6	<3	<4	256	192	384
	4	3-2	6	8	768	512	2048
	4	4-3	12	8	128	256	2048
	5	3-3	8	12	1024	768	3072
In	5	4-5	16	16	512	512	8192
saline	6	3-4	24	24	384	768	4096
	6	4-7	24	16	512	512	4096
	9	3-5	32	32	128	128	2048
	9	4-4	24	32	256	256	8192
	14	4-2	24	32	64	96	2048
	14	4-6	16	24	64	32	2048
	3	4-1	<3	<4	384	192	768
	4	3-7	16	16	192	192	3072
	4	9-0	12	24	784	256	4096
	5	3-8	24	32	256	512	1536
In	5	9-4	32	32	1024	512	6144
paraffin	6	3-9	32	32	768	512	4096
oil	6	9-6	48	32	256	512	3072
	9	4-0	32	48	768	512	3072
	9	9-7	48	32	1024	2048	8192
	14	9-1	32	64	2048	1024	8192
	14	9-5	64	48	1024	2048	8192

* Rt. refers to the right leg, Lt. to the left leg. The right foot was injected with 0.25 mg., the left with 1.25 mg. of vaccine.

nodes and antibody response. It was found that the highest titers in the lymph nodes preceded those at the site of injection (Table I). Similarly the ratios between lymph node and foot pad titers were highest early in the experiment when antibody production was highest. This is shown by a brief tabulation derived from the antigen-in-saline experiments of Table I:

Days	Average ratio between lymph node and foot pad titers	Average titer of lymph node extract	Average titer of blood serum
3	67	224	384
4	59	416	2048
5	64	704	5632
6	20	544	4096
9	7	192	5120
14	5	64	2048

These findings are consistent with a primary appearance of the antibody in the popliteal lymph node, but hard to reconcile with a primary appearance at the site of injection.

2. The Relative Antibody Titers in the Peritoneal Fluid and Peritoneal Cell Extract after Intraperitoneal Introduction of Various Antigen Combinations

The other avenue of approach to our problem that presented itself grew from the well known fact that intraperitoneal injection of antigen is followed first by a predominantly granulocyte and later by a predominantly macrophage response. If antibody were synthesized in the granulocytes or macrophages, its presence should be directly demonstrable in these cells during the period of antibody production, as it was demonstrated in the lymphocytes (2).

Five series of experiments were performed: 2 mg. of dysentery vaccine were given intraperitoneally: (1) in 10 cc. of saline; (2) in 10 cc. of saline containing 4 gm. of graphite; (3) in 10 cc. of saline containing 2 gm. of aleuronat; and (4) in 10 cc. of saline-in-paraffin-oil emulsion. In the fifth experiment 2 mg. of typhoid vaccine were given intraperitoneally in 10 cc. of saline-in-paraffin-oil emulsion.

As hardly any fluid was found in the peritoneal cavity after the injection of these materials, 20 cc. of saline containing 0.5 per cent of oxalate were injected intraperitoneally shortly before the animal was sacrificed. But even with this measure we often recovered not more than 10 cc. of fluid; in only two instances did we collect more than 20 cc.

The results of these experiments are given in Table II. It can be seen that the cellular response in the peritoneal cavity (at the site of injection) was most marked after injection of antigen in oil, or antigen with aleuronat; it was least marked after antigen in saline. After the injection of antigen with oil, graphite, or saline we found a sharp rise in granulocytes followed by a rapid decline in these cells; the latter was well under way 3 days after injection. After aleuronat the granulocyte response lasted considerably longer. The largest number of macrophages was found in the experiments with antigen in oil and with aleuronat.

The antibody response as manifested by the serum titer was most marked during the period of observation in the experiments with saline, graphite, and aleuronat; it was definitely delayed in the antigen-in-oil experiments though

TABLE II

Comparison of Antibody Titers in Peritoneal Fluid and Peritoneal Cell Extract after Intraperitoneal Injection of Various Antigen Combinations

Antigen	Time after injection of antigen	Rabbit No.	Peritoneal fluid recovered	Total No. of leukocytes	Granulocytes	Macrophages*	Granulocytes	Macrophages*	Titer of		
									Peritoneal fluid	Peritoneal cell extract	Blood serum
	days		*cc.*	*per c.mm.*	*per cent*	*per cent*	*per c.mm.*	*per c.mm.*			
2 mg. dysentery vaccine in 10 cc. saline	1	1-11	10		93	7			<3	<3	32
	2	8-8	10	7,100	22	78	1,562	5,538	<3	<3	24
	3	8-9	8		41	59			<3	<3	384
	5	1-09	10	1,100	25	75	275	825	48	<3	1576
	7	1-10	17	1,600	29	71	464	1,136	256	<3	6184
2 mg. dysentery vaccine in 4 gm. graphite in 10 cc. saline	1	6-5	10	13,400	90	10	12,060	1,340	3	<4	6
	2	3-0	5	23,350	69	31	16,912	7,238	4	<4	24
	2	8-4	10	20,200	79	21	15,958	4,242	3	<3	24
	2	1-13	13	16,800	79	21	13,272	3,528	<3	<3	24
	3	6-4	10	17,400	72	28	12,528	4,872	3	<4	64
	5	6-8	10	4,950	81	19	4,010	940	64	<4	1024
	9	8-5	10	1,150	20	80	230	920	24	<3	2048
2 mg. dysentery vaccine in 2 gm. aleuronat in 10 cc. saline	1	1-22	12	19,500	87	13	16,905	2,535	8	<3	<24
	2	1-23	15	21,600	80	20	17,280	4,320	4	<3	<24
	3	1-20	12	23,300	86	14	20,038	3,262	16	<3	64
	3	1-21	13	49,800	82	18	40,836	8,964	4	<3	96
	5	1-24	5	26,900	67	33	18,023	8,877	192	<3	2048
	7	1-25	11	40,600	49	51	19,894	20,706	256	8	6184
2 mg. dysentery vaccine in 10cc. paraffin-oil-Falba emulsion	1	6-6	28	47,200	91	9	42,952	4,248	0	<4	6
	1	8-3	8		83	17			<3	<3	<24
	2	2-9	10	60,300	76	24	45,828	14,472	4	<4	32
	2	8-0	10	43,600	66	34	28,776	14,824	<3	<3	64
	3	3-1	8	67,200	22	78	14,784	52,416	8	<4	64
	5	6-7	10	17,250	55	45	9,488	7,762	16	<4	384
	7	6-9	10	17,800					32	<4	1024
	7	8-1	12	12,600	52	48	6,552	6,048	48	<3	512
	9	8-2	13	10,600	27	73	2,862	7,738	32	<3	512
2 mg. typhoid vaccine in 10 cc. paraffin-oil-Falba emulsion	1	1-26	22	28,500	91	9	25,935	2,565	<4	<3	16
	2	1-27	13	34,100	89	11	30,349	3,751	<4	<3	32
	3	1-28	13	19,000	69	31	13,110	5,890	16	<3	256
	5	1-29	19	16,100	51	49	8,211	7,889	32	<3	768

* The cells counted as macrophages include a small number of small lymphocyte-like cells. As they amounted to an average of not more than 3 per cent, no attempt was made to determine whether they were small macrophages or lymphocytes.

the latter, with the aleuronat experiments, showed the best macrophage response. The typhoid experiments were not materially different from the dysentery experiments.

Considering the antibody content of the peritoneal fluid, it is shown in Table II that no antibody was found in the cell extracts of 30 of 31 animals. Only one rabbit showed a titer of 1:8. As the supernatant fluid of this specimen contained 32 times, and the blood serum 773 times as much antibody, it appears that in this animal some supernatant fluid may have been left with the sediment; it can be calculated from these figures that a minute fraction of the supernatant fluid left with the cell extract would easily account for the titer.

TABLE III

Antibody Titers in Peritoneal Fluid, Peritoneal Cell Extract, and Blood Serum after Intravenous Injection of Dysentery Antibody and Subsequent Intraperitoneal Injections of Paraffin-Oil-Falba

Time after injection	Rabbit No.	Peritoneal fluid recovered	Total No. of leukocytes	Granulocytes	Macrophages*	Granulocytes	Macrophages*	Titer of		Blood serum
								Peritoneal fluid	Peritoneal cell extract	
days		*cc.*	*per c.mm.*	*per cent*	*per cent*	*per c.mm.*	*per c.mm.*			
1	1-47	15	20,100	79	21	15,879	4,221	8	<3	96
1	1-48	12	38,500	84	16	32,340	6,160	4	<3	96
3	1-43	16	15,100	59	41	8,909	6,191	4	<3	128
3	1-44	18	16,800	44	56	7,392	9,408	4	<3	128
5	1-45	18	18,500	23	77	4,255	14,245	16	<3	128
5	1-46	19	20,200	24	76	4,848	15,352	16	<3	128
7	1-55	20	2,400	5	95	120	2,280	32	<3	96
7	1-56	11	6,200	2	98	124	6,076	24	<3	128
9	1-57	20	5,600	2	98	112	5,488	8	<3	64

* Compare footnote of Table II.

As to the supernatant fluid, it can be seen that its titers roughly paralleled those of the blood serum except that the latter were on the average 20 times as high as the former. This difference in titer was obviously largely due to the dilution of the exudate by the intraperitoneal injection of 20 cc. of saline-oxalate solution shortly before the animals were sacrificed. In some animals (6-5, 1-20, etc.) the titer in the supernatant fluid may have been higher than in the blood serum.

Considering the origin of the antibody in the peritoneal fluid it seemed that it was fully explained by secondary concentration here of antibody that was synthesized elsewhere. In order to test this hypothesis, a series of 9 rabbits were given intravenous injections of dysentery antibody produced by active immunization of rabbits. Each animal received 30 cc. of antidysentery serum

showing a titer of 1:1024. After injection of this serum, 10 cc. of paraffin-oil-Falba emulsion were introduced intraperitoneally, and at different intervals thereafter the rabbits were sacrificed and studied precisely as in the preceding series of experiments. The results of this study are given in Table III.

If we compare Tables II and III, we find a remarkable similarity between the relative titers in the peritoneal fluid and in the blood serum. Again the titers of the supernatant fluid roughly parallel those of the blood serum; again the latter are on the average 20 times as high as the former; again no antibody is contained in the cell extract. These data seem to prove that the low titers of the peritoneal fluid and also of the foot pads which were observed in our previous experiments, are satisfactorily explained by fixation at the site of inflammation; there remains nothing to suggest that either the granulocyte or the macrophage was instrumental in the synthesis of these antibodies.

COMMENT

The reticuloendothelial theory of antibody formation has been critically reviewed elsewhere (10). It has been pointed out that this theory rested chiefly on the fact discovered by Metchnikoff 60 years ago that these elements engulfed and digested formed antigenic material. That phagocytosis and digestion resulted in antibody synthesis was merely speculation. No evidence has ever been presented to show that antibodies are products of this function; the products of digestion of the macrophages have never been identified.

In the present series of experiments it has been shown that the granulocytes and macrophages did not develop significant quantities of agglutinin against dysentery or typhoid bacilli whilst this antibody was formed. This was particularly obvious in our intraperitoneal experiments where the granulocytes and macrophages could be studied in an isolated way. As the quantity of cells available for serologic analysis was about 10 times as large as in our experiments on efferent lymph reported previously (2) and therefore it would have been possible to detect very low concentrations of antibody contained therein, our negative results must be regarded as significant.

Whether or not macrophages can synthesize antibody other than agglutinin cannot be decided at present. The fact that the cytoplasm of these cells is acidophilic rather than basophilic, speaks against protein synthesis here, for it seems to be well established that where protein is synthesized the cytoplasm is basophilic due to the presence of large quantities of ribosenucleic acid (11).

When speaking of protein synthesis, we mean the anabolic processes by which the globulins are formed. We do not wish to imply that we think that the granulocytes and macrophages may not have some other function in antibody formation; in fact, we do not doubt that these cells play an important rôle in the complicated process which eventually culminates in the output of antibody, whether by the killing of microorganisms, or the destruction of toxins,

or through dissolving particulate antigens (the only function of these cells that has been actually observed), or through retention of antigenic material which otherwise would be excreted or destroyed elsewhere in the body (9). For these reasons alone, both granulocytes and macrophages have their places in antibody formation.

SUMMARY

Following the injection of dysentery antigen in saline or in saline-in-paraffin-oil emulsion into the pad of the rabbit's hind foot, considerable quantities of antibody were recovered from the popliteal lymph node, while the tissue at the site of injection, containing many granulocytes and numerous macrophages, revealed only insignificant quantities of antibody.

Following the injection of various dysentery and typhoid antigen combinations into the abdominal cavity, no antibody was found in the isolated granulocytes and macrophages of the peritoneal exudate, while the supernatant fluid revealed titers that roughly paralleled those of the blood serum. Similar results were obtained when animals were injected first with antibody intravenously, and subsequently with an unspecific irritant intraperitoneally. The presence of antibody in the supernatant fluid was, therefore, interpreted as being due to secondary concentration (fixation) in an inflamed area.

These findings together with the previously described observations on the lymphocyte seem to show that the macrophage does not synthesize agglutinins against dysentery or typhoid bacilli.

BIBLIOGRAPHY

1. Ehrich, W. E., and Harris, T. N., *J. Exp. Med.*, 1942, **76,** 335.
2. Harris, T. N., Grimm, E., Mertens, E., and Ehrich, W. E., *J. Exp. Med.*, 1945, **81,** 73.
3. Ehrich, W. E., *Ann. New York Acad. Sc.*, in press.
4. Dougherty, T. F., Chase, J. H., and White, A., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57,** 295.
5. Dougherty, T. F., White, A., and Chase, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59,** 172.
6. White, A., and Dougherty, T. F., *Ann. New York Acad. Sc.*, in press.
7. White, A., and Dougherty, T. F., *Endocrinology*, 1945, **36,** 207.
8. Editorial, *J. Am. Med. Assn.*, 1945, **128,** 1232.
9. Ehrich, W. E., Halbert, S. P., Mertens, E., and Mudd, S., *J. Exp. Med.*, 1945, **82,** 343.
10. Ehrich, W. E., and Harris, T. N., *Science*, 1945, **101,** 28.
11. Mirsky, A. E., in Advances in Enzymology and Related Subjects of Biochemistry, (F. F. Nord and C. R. Werkman, editors), New York, Interscience Publishers, Inc., 1943, **3,** 1.