Adjuvant Effect of Diffusion Chambers on Soluble Antigens.* (27893)

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Diffusion chambers of the type pioneered by Algire(1) are widely used for functional and morphological studies on tissue transplants. When equipped with filters of the proper pore size such chambers allow for diffusion of soluble substances while protecting the transplants against attack by cells of the host animal. Chambers have been used by others and by us(2,3) in studies on antibody formation by transplanted cells. Control experiments carried out during our work have shown that insertion of a diffusion chamber into the peritoneal cavity of an animal may exert a strong adjuvant effect on the immunogenicity of soluble antigens. The data to be presented illustrate this adjuvant effect and define some of the conditions required for its production.

Materials and methods. The mice used were of the DBA/1 strain, bred in this laboratory from an original stock obtained through the Jackson Memorial Laboratory. Rats were of the Wistar strain and were obtained commercially. Control and experimental animals were matched according to age and sex. Sera were prepared from blood specimens obtained by puncture of the infra-orbital sinus; sera were stored at −20°C for no longer than 4 weeks prior to titration. Antibody titrations were carried out using the tanned cell agglutination technic(4); sheep red cells were employed, and saline buffered at pH 7.6 and containing 1% normal rabbit serum served as diluent. The results were analyzed statistically and the significance of observed differences was tested with the aid of Student's "t" test(5). Ferritin prepared from horse spleens(6) had been recrystallized 5 times; hemocyanin was prepared by repeated precipitation at its isoelectric point from the hemolymph of *Busycon canaliculatum*. Both antigens gave but a single line of precipitation when tested in Ouchterlony plates with their respective antisera.

Chambers used in mice were made of lucite cylinders 5 mm high with an outer diameter of 13 mm and an inner diameter of 9.6 mm. Those used in rats were of the same height, but had an outer diameter of 26.5 mm. The membranes used in all experiments were of 0.1 μ pore size and 0.5 or 1 inch diameter, respectively. Some of the earlier experiments were done with membranes of these specifications obtained from the Millipore Co.; identical results were obtained in later experiments with Polypore membranes of the same specifications obtained from Gelman Instrument Co., Chelsea, Mich. The filters were glued to the lucite rings with Millipore Cement No. 1. A filling hole through the lucite ring was closed after filling of the chamber with a peg of lucite from Williams Gold Refining Co., Buffalo, N. Y., according to the procedure employed by Amos(7). The assembled chambers and the pegs were sterilized by exposure to ultraviolet light. Ether was employed for anesthesia of the animals and the chambers were inserted into the peritoneal cavity through a lateral incision which was closed with 11 mm wound clips. Mortality was negligible until after several bleedings had been performed.

Experimental. Since preliminary and cursory observations made during our work on antibody formation had suggested that diffusion chambers may exert an adjuvant effect on soluble antigens, a systematic titration of the antigenicity of one antigen, ferritin, was carried out in mice. The experimental animals (Group I) received intraperitoneally diffusion chambers charged with 0.2 ml amounts of varying dilutions of ferritin, the control animals (Group II) were injected once intraperitoneally with 0.2 ml of a different set of ferritin dilutions. The results (Table I), clearly demonstrate the adjuvant effect of the chambers. Five μg of ferritin N was the minimal dose which given

* This work was supported in part by grant from Nat. Inst. of Allergy and Infect. Dis., Nat. Inst. Health, USPHS.

TABLE I. Antibody Response of Mice to Ferritin Injected or Given by Chamber.

Group	Dose (μg N in 0.2 ml)	Mean ± 2 standard errors of titers* after 1	2	3 weeks
I In chamber IP	.008	<1.0 (13)†	<1.0 (11)	<1.0 (8)
	.04	<1.0 (11)	<1.0 (11)	<1.0 (8)
	.1–0.2	3.9 ± .66 (28)	3.1 ± .70 (36)	2.7 ± .72 (29)
	.5–1.0	4.1 ± .74 (22)	4.5 ± .72 (20)	4.7 ± 1.06 (16)
	5	6.2 ± .66 (48)	5.7 ± .62 (46)	5.6 ± .60 (22)
	25	7.5 ± .98 (19)	8.1 ± 1.00 (18)	6.9 ± .98 (15)
II Injected IP	5	1.0 ± .52 (39)	<1.0 (34)	<1.0 (32)
	20–25	4.0 ± .72 (32)	3.6 ± .56 (45)	2.4 ± .50 (43)
	50	3.7 ± .28 (43)	3.8 ± .66 (50)	3.8 ± 1.06 (20)
	100–200	5.7 ± .88 (20)	6.5 ± 1.02 (16)	6.7 ± 1.02 (14)
	500	6.5 ± 1.04 (12)	7.1 ± .80 (12)	6.7 ± .88 (12)

* No. of tube containing limiting dilution of antibody in a serial 2-fold dilution series started with serum diluted 1:25.

† No. of mice in group.

by intraperitoneal injection resulted in a detectable antibody response. In contrast, less than 0.1 μg ferritin N sufficed when ferritin was administered in a chamber. A comparison of the responses to other doses of antigen shows that to obtain a given level of antibody from 25 to 125 times more ferritin was required by injection than by the chamber technic.

The results given in Table I further show that mice in the experimental and control groups had attained maximum titers of antibody on or before the 7th day, and that those given the larger doses within each group maintained these antibody levels for at least another 2 weeks. Those mice which received 5 μg of ferritin N in chambers still possessed essentially unchanged antibody titers when bled 10 weeks later.

Similar though less extensive experiments were conducted with rats. Whereas intraperitoneal injection of 20 μg ferritin N, even when repeated once, led to no more than traces of antibody formation, antibody titers ranging from 1:20 to 1:640 were attained in all of 10 rats given chambers containing 5 μg ferritin N.

Another antigen, hemocyanin, evoked only trace amounts of antibody when injected intraperitoneally into rats in amounts of 50 μg N. When given in diffusion chambers 10 μg N led to production of antibody titers ranging from 1:80 to 1:1280 in 11 of 13 rats.

One possible explanation of the mechanism by which the chambers enhanced the antigenicity of soluble protein antigens was that of the chamber functioning as a depot from which antigen escaped slowly over a prolonged period of time. In apparent conflict with this possibility was the finding that ferritin appeared in the serum of mice in detectable amounts within one hour after it had been administered either in a chamber or by injection into the peritoneal cavity and that blood levels of the antigen remained about the same for the next 24 hours regardless of mode of administration. Also, the adjuvant effect was quantitatively the same whether an arbitrarily selected test dose of 5 μg ferritin N was given in a chamber or by intraperitoneal injection into mice which had been equipped just prior to their injection with chambers filled with saline (Groups 1 and 2, Table II). The saline used in the chambers was clearly not responsible since chambers filled with 0.2 ml mouse serum, in each case the serum of the prospective recipient of the chamber, were again found to be highly effective in conjunction with an intraperitoneal injection (Group 3, Table II).

Results of additional experiments ruled out surgery or anesthesia as causes of the adjuvant effect (Group 4). Individual components of the chambers, or complete but unassembled chambers, displayed a modest stimulatory effect on antibody production (Group 5) which was far inferior to the adjuvant effect described above. Empty as-

TABLE II. Ajuvant Effect of Diffusion Chambers and Components.

Group			Mean ± 2standard errors in titers after 1	2	3 weeks
1	Ferritin in chamber		6.2 ± .66 (48)	5.7 ± .62 (46)	5.6 ± .60 (22)
2	Saline " "	Ferritin IP	5.3 ± .62 (59)	5.0 ± .72 (38)	5.2 ± .82 (32)
3	Serum " "	" "	5.7 ± .96 (14)	4.9 ± 1.02 (10)	
4	Sham operated	" "	1.1 ± .52 (34)	1.4 ± 1.16 (36)	<1.0 (12)
5	Chamber components	" "	1.9 ± .62 (44)	2.3 ± .62 (36)	2.5 ± .88 (15)
6	Empty chambers	" "	2.3 ± .78 (32)	2.0 ± .72 (32)	1.9 ± 1.00 (17)
7	Saline in sealed chamber	" "	2.6 ± 1.28 (13)	2.1 ± 1.16 (11)	2.0 ± 1.20 (10)
8		" SC	1.2 (14)	1.4 (14)	1.4 (14)
9	Saline in chamber	" "	2.9 ± .78 (14)	2.2 ± .62 (14)	1.8 (14)

See legend to Table I.

sembled chambers (Group 6), in contrast to saline- or serum-filled chambers, were quite ineffective. Similarly, saline-filled chambers which were treated with adhesive so as to seal the filter pores, were devoid of adjuvant action and no better than chamber components. It was finally found that adjuvance with saline-filled chambers failed to occur if the chamber was inserted intraperitoneally and the antigen was injected subcutaneously (Groups 8 and 9).

Further inquiry as to how chambers charged with antigen, or "wet" but not "dry" chambers, together with an injection of antigen, enhanced the immune response led to observations and experiments presented below. It was found that empty chambers, inserted intraperitoneally, contained trace amounts of 0.05 ml or less of ascitic fluid when removed from the animals after 24 hours. After 4 days chambers that were inserted empty contained an average of 0.1 ml ascitic fluid and after 11 days they were full, except for a small air bubble, containing an average of 0.33 ml ascitic fluid. At this time the protein content of the fluid recovered from chambers inserted empty was exactly the same as that of chambers which had originally been filled with saline. The 5.7-5.8 mg N/ml found was the same amount as that in ascitic fluids evoked by injection of complete Freund's adjuvant (unpublished data), and averaged approximately 60% of the serum protein.

The grossly visible reaction to chambers charged with saline, with antigen, or inserted empty was minimal. Generally the chambers were found free in the peritoneal cavity, even after 3-8 weeks. Occasionally one filter surface was covered by a thin, transparent membrane; on rare occasions this membrane enveloped the whole chamber. Hemorrhagic and edematous areas adjacent to the membrane filter surfaces, often seen in reactions to diffusion chambers which had been charged with tissues or had become contaminated with bacteria, were not observed. To determine how long after insertion the adjuvant effect of chambers would persist, mice which had received saline-filled chambers 8 weeks earlier were injected intraperitoneally with 5 μg ferritin N. Their antibody titers measured 1-3 weeks later were identical with those of mice in Groups 5-7 (Table II) and significantly lower than those of Groups 1-3 (Table II). Thus chambers which had been in the mice for 8 weeks appeared to be no more effective than empty chambers or chamber components.

In further experiments mice were injected with a standard dose of hemocyanin (5 μg N) intraperitoneally. The results (Table III) show that a saline-filled chamber inserted on the day of injection exerted an adjuvant effect (Groups 1 and 2). An even better enhancement of the antibody response against this antigen was obtained when the antigen was placed into the chamber (Group 3). If new chambers were filled not with saline but with ascitic fluid removed from chambers which had been in other mice for periods of 3-8 weeks the response to hemocyanin was greatly enhanced (Group 4). Thus the failure of "old" chambers to mediate the adjuvant effect could not be attributed to their content of ascitic fluid. Using

TABLE III. Persistence of Adjuvant-Action by Chambers in Mice.

Group	Treatment	Mean ± 2 standard errors of titers† after 4 weeks
1	Normal mice, injected IP	2.4 ± .72 (27)
2	Saline-filled chamber & IP injection, same day	4.7 ± .62 (20)
3	Hemocyanin-filled chamber	6.1 ± .66 (10)
4	Ascitic fluid-filled chamber* & IP injection, same day	5.6 ± 1.62 (7)
5	IP injection into mice carrying chambers for 3 weeks	1.4 ± .48 (36)
6	IP injection & chambers removed from mice after 3-8 weeks, same day	3.6 ± .80 (26)

All mice received 0.2 ml of a solution of hemocyanin containing 5 μg N.

* Ascitic fluid harvested from chambers after 3-8 weeks, placed into new chambers in 0.2 ml amounts.

† Number of tube containing limiting dilution of antiserum in a 2-fold dilution series starting with serum diluting 1:100.

hemocyanin as the test antigen, no adjuvant effect could be detected in mice injected 3 weeks after they had received chambers which originally were filled with saline or ferritin (Group 5). In this group the prior response in some of the mice to ferritin appeared to be irrelevant to their subsequent response to hemocyanin. Finally (Group 6) it could be shown that chambers which were removed from mice 3 to 8 weeks after their implantation and, after superficial wiping, were implanted in new mice, were moderately effective as adjuvants. The differences in response of Groups 1 and 6 on the one hand and of Groups 2 and 6 on the other hand were statistically significant on the 5% level of probability. Results of similar experiments with rats indicated that the adjuvant effect of chambers persisted for longer periods of times (8 weeks) in these animals. Since larger chambers were used in the rat experiments it is not clear whether this apparent difference can be attributed to species differences or to technical factors related to chamber size.

Discussion. The data presented here show that minute amounts of soluble antigens, insufficient to elicit antibody when given by intraperitoneal injection, give rise to considerable amounts of antibody when administered in diffusion chambers. This obviously implies that experiments on antibody formation by tissues or cells transplanted in diffusion chambers require rigid controls to eliminate the possibility of an active immunization of the host animal by trace amounts of transferred antigen.

The mechanism by which chambers exert their adjuvant effect remains obscure. Irritation by the chamber components plays at best a subordinate role (Table II). Two observations appear to contradict the concept of function of chambers as depots from which antigen diffuses slowly and thus more effectively. The similar rates of antigen escaping into the blood after administration of antigen by injection or by chamber may not be a serious obstacle since such escaping antigen may not be of primary relevance to the antibody response. More difficult to explain is the similar effectiveness of antigen in the chamber and of antigen given by injection after insertion of a saline-filled chamber. It would be necessary to postulate that sufficient antigen diffuses into the chamber, presumably at a rapid rate and within a short period after injection, and that this antigen is then released slowly. A decrease in rate of exchange of fluids might be the result of clogging of the membrane pores with time. The failure of empty chambers to exert the adjuvant effect might be related to resistance against the entry of fluid exerted by air trapped in the chamber and its pores. It has been suggested(8) that it might be necessary for such air to become dissolved to clear the passage.

One cannot ignore an alternate hypothesis, however, namely that of an attraction by the chamber for cells of a type which would channel the antigen most effectively into productive pathways. It could be assumed that moist filter membranes provide a suitable site for the accumulation and proper function of such cells whereas dry membranes, such as those on empty chambers or on membranes with sealed pores, might fail to serve this purpose.

Summary. Data have been presented which show a marked adjuvant effect of diffusion chambers made with filters of 0.1 μ pore size on the antigenicity of 2 soluble an-

tigens, hemocyanin and ferritin, in rats and mice. This effect occurs not only when antigen is administered in the chamber but also after insertion of a saline-filled chamber followed by intraperitoneal injection of the antigen. Chamber components were shown to be not responsible for the enhanced antibody production. The possible mechanism has been discussed in terms of a depot function and also in terms of the cellular response on and around the chambers. Also discussed were the implications of these findings on studies of antibody formation in diffusion chambers.

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Received July 30, 1962. P.S.E.B.M., 1962, v111.

Antimitotic Action of 5-(2'-Bromoacetamido)-Uracil on the Ehrlich Ascites Tumor.* (27894)

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Since the first reports of the antimetabolic effects of 5-fluorouracil (5-FU)† by Heidelberger *et al.*(1,2), there has been considerable interest in this and other halogenated pyrimidines. The antimetabolic activity of these compounds has been attributed largely to their ability to interfere with the deoxyribonucleic acid synthesis of several lower organisms and transplanted tumors of animals. In related studies, Visser(6) and his co-workers tested 5-(2'-bromoacetamido)-uracil (BAU)† on wild and mutant strains of *Neurospora* and found that this compound inhibited growth of the wild type. In this paper are reported observations on the cytological effects of BAU on Ehrlich ascites tumor cells and the results are compared with those obtained with 5-fluorouracil.

Materials and methods. A mouse tumor, Ehrlich ascites tumor (hypotetraploid strain), was used to study the effects of BAU and 5-FU. The ascites tumor was transmitted serially in Swiss/HaICR mice weighing 18-20 g, fed on a stock diet of Purina chow and water. Intraperitoneal injections of freshly prepared aqueous solutions or suspensions of chemicals usually were begun on the fifth day after transplantation of the tumor, when the tumor was well established and active proliferation of the tumor cells was taking place in the peritoneal cavity. At various intervals, samples of tumor ascites were removed by peritoneal puncture, squashed with acetic dahlia, and examined cytologically. The numerical data were calculated on the basis of approximately 500 interphase and dividing cells for every sample.

Results. 1. *Effects of 5-(2'-bromoacetamido)-uracil (BAU).* Serial cytological studies were made of tumor cells obtained from mice after single injections of BAU on the fifth day after tumor transplantation. The following numbers of mice were employed at each dose level: 10 received 1 mg; 11, 2 mg; and 3, 3 mg. The life span of the animals receiving 1 and 2 mg was the same as that of the controls, 10-12 days after

* This work was supported in part by USPHS grant.

† The authors gratefully acknowledge the generosity of Dr. D. W. Visser, Univ. of Southern California, Los Angeles, who supplied the sample of BAU, and Dr. M. J. Shiffrin, Hoffmann-LaRoche, Inc., Nutley, N. J., who supplied the sample of 5-FU.