PRIMARY IMMUNIZATION OF LYMPH NODE CELLS IN MILLIPORE CHAMBERS BY EXPOSURE TO HOMOGRAFT ANTIGEN*

By HAROLD F. DVORAK‡ and BYRON H. WAKSMAN,§ M.D.

(From the Department of Pathology and the Neurology Research Laboratory, Massachusetts General Hospital, Boston)

Plates 1 and 2

(Received for publication, February 7, 1962)

One of the more troublesome difficulties confronting the student of homograft sensitivity is the inability to repeat *in vitro* the immune and immunizing events routinely produced in the intact animal. To repeat such experiments in an isolated system requires the successful culture of functioning lymph node cells and this has generally not been possible.

In an attempt to isolate the immune event, Medawar (1) and again Scothorne and Nagy (2), Weaver *et al.* (3), Trowell (4), and Harris (5) cultured "target" cells with specific immune lymphoid tissues but were consistently unable to demonstrate anything comparable to the homograft reaction seen in the intact animal. More recently, however, Govaerts (6) found that renal cortical cells, grown *in vitro* with thoracic duct lymphocytes and serum obtained from specifically immune animals, became "retracted, round, and somewhat agglutinated" with granular, vacuolated cytoplasm. Rosenau and Moon (7) have confirmed this work by showing that L strain cells derived from C3H mice develop cytopathologic changes and are destroyed by specifically sensitized Balb/c lymphocytes in tissue culture.

The problem of immunization *in vitro* has been studied mainly with regard to antibody formation. Here, while secondary stimulation of cells sensitized in the intact animal has been successful, primary sensitization of previously untreated cells has proven difficult (8).

A useful approach to the problem of long term culture of functioning lymphoid cells was taken by Algire (3, 9) who constructed diffusion chambers of membrane filters which allowed the passage of fluids, salts, and proteins, but which were impervious to cells. In chambers constructed of such materials and implanted in the peritoneal cavities of "carrier" animals, lymph node cells were found to survive and function for periods of several weeks.

Algire and his collaborators have shown that immune lymphoid cells placed in chambers with appropriate antigenic "target" cells are able to destroy the "target" cells, presumably by

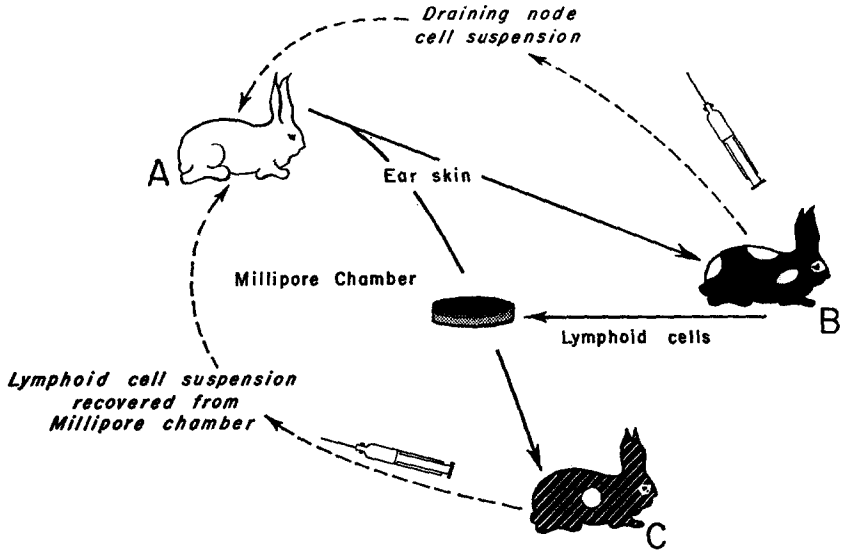
* This study was supported by Grant No. E-1257 of the National Institute of Allergy and Infectious Diseases.

‡ Postsophomore Fellow of the National Institutes of Health.

§ National Neurological Research Foundation Scientist.

a homograft type of reaction. Non-immune cells had little or no effect. More recently Holub (10, 11), using a modification of Algire's technique which allows recovery of fluid and lymphoid cells from chambers, has succeeded in initiating antibody production in normal lymphoid cells exposed to protein and bacterial antigens. Capalbo *et al.* (12), using the same approach, have been able to elicit the production of red blood cell agglutinins.

It was the purpose of the present investigation to extend these findings to the delayed form of hypersensitivity by inducing primary immunity in normal lymphoid cells exposed to homograft antigens in Millipore diffusion chambers.



TEXT-FIG. 1

The induced immunity was assessed by the use of the "transfer reaction" described by Brent *et al.* (13, 14). These authors showed that lymphoid cells of one animal, sensitized against homografts from a second animal, produced a local tuberculin-like response when injected intradermally in the antigen donor. This reaction can be made to appear in both guinea pigs and rabbits, but in rabbits is more violent and is elicited with fewer cells. Because skin homografts afford an antigen which is non-diffusible through Millipore filters and omnipresent in skin test sites and because few cells are required to give strong lesions, the "transfer reaction" proved to be a convenient tool for our purposes.

Materials and Methods

The experimental plan is presented diagramatically in Text-fig. 1. Normal lymphoid cells from one rabbit (*B*) were placed in Millipore diffusion chambers with skin slices from a second

rabbit (*A*). The chambers were then implanted in the peritoneal cavities of indifferent third rabbits (*C*) for culture. At appropriate intervals cells were harvested and assayed for immunity using the "transfer reaction" as a test system; *i.e.*, recovered cells were injected intradermally in the antigen (skin slice) donor and the injection sites examined at 24, 48, and sometimes at 72 hours for the appearance of a delayed skin reaction. Appropriate controls were established. Along with the preparation of chambers, skin grafts from the antigen donor (*A*) were implanted in the rabbit which donated the lymphoid tissue (*B*). The node draining the implantation site in this animal (*B*) was recovered simultaneously with the chambers and a cell suspension prepared from it was likewise injected intradermally in the antigen donor (*A*). Thus, it was possible to run *in vivo* and semi-*in vitro* experiments in parallel, using the same animal pair.

Animals.—Adult female New Zealand and Dutch rabbits were used throughout, the former as a source of homograft antigen and the latter as a source of normal and immune lymphoid tissue. The use of two strains of rabbits was based on preliminary experiments which indicated that New Zealand rabbits, while not inbred in the strict sense, were too closely related to give consistent "transfer reactions" when sensitized against each other. New Zealand, Dutch, and a few Flemish Giant rabbits were used as chamber "carriers." The identity of the "carrier" rabbit did not affect the results obtained. All animals were caged individually and kept on an *ad libitum* diet of standard Purina laboratory chow and water.

Diluent.—For all manipulations carried out in the present study, cells were suspended in a diluent of chilled Hanks' solution to which penicillin (100 units/ml) and streptomycin (0.125 mg/ml) were added.

Antigen.—Ear skin served as the source of homograft antigen. New Zealand and Dutch rabbits were anaesthetized with nembutal, the inner surface of the pinna shaved and washed with 70 per cent alcohol, and thin slices of skin cut with a sharp scalpel. Skin so obtained consists of epidermis plus a thin layer of dermis and resembles grafts cut with a dermatome. The skin slices were transferred to the diluent. In one experiment an epidermal cell suspension was prepared from New Zealand ear skin with trypsin following the technique of Billingham (15) and a known number of these cells used as antigen.

Lymphoid Tissues.—Retroscapular and popliteal lymph nodes and spleens were recovered from Dutch rabbits under nembutal anaesthesia using semi-sterile technique. They were immediately transferred to chilled diluent and maintained at 10°C until ready for use. They were then minced with a sharp pair of iris scissors. In some cases a cell suspension was made by washing the mince with diluent and this was counted, spun down, and diluted appropriately. The residue left over, after the mince had been washed and the suspension prepared, was also planted in chambers.

Chambers.—Diffusion chambers were constructed of nylon-reinforced Millipore filters (85 micra thick, pore size 0.45 ± 0.05 micra) and lucite rings (22 mm outside diameter, 19 mm inside diameter, and 3 mm height). Disks of filter material 24 mm in diameter were glued to one side of such lucite rings with Millipore cement, preparation 1, and gas-sterilized in plastic bags. For use chambers were floated on cold diluent, filled with appropriate tissues, and a second disk of filter material, the roof, glued to the open end of the lucite ring. The result was a cell-tight, fluid-permeable chamber which could be kept in cold diluent until preparations were completed for peritoneal implantation.

Handling of Chambers and Their Contents.—Experimental chambers contained three 5 × 5 mm slices of New Zealand ear skin or, in one case, 1.25 million free epidermal cells. Control chambers generally contained three slices of Dutch ear skin or in rare instances, no skin at all. All chambers were inoculated with a mince, counted cell suspension, or residue from a counted cell suspension of Dutch lymphoid tissues. A cell suspension obtained from two popliteal and two retroscapular lymph nodes was sufficient for six chambers (13 to 14 million

viable cells per chamber) and the remaining residue for at least two chambers. Three lymph nodes (two popliteals and one retroscapular) or one spleen afforded sufficient mince for eight chambers. This corresponds to a quantity of mince for each chamber from which 10 to 20 million viable cells can be obtained in suspension. Experiments were done in units of eight chambers, generally four experimental and four control. Chambers were filled to approximately three-quarters of their capacity with diluent to prevent desiccation of cells before the forces of diffusion had time to operate.

"Carrier" rabbits were anaesthetized with nembutal and a 5 cm longitudinal midline incision made. Peritoneal folds were retracted and chambers implanted in each abdominal quadrant; *e.g.*, four chambers per "carrier" animal, two experimental and two control. Careful initial placement prevented chambers from sticking together with a resulting death of chamber contents. The incision was closed in two layers with silk and wound clips.

Chamber Fate and Recovery.—Chambers placed in the peritoneal cavities of "carrier" rabbits gradually acquired a monolayer sheath of host polymorphonuclears and eventually of chronic inflammatory cells and fibroblasts, if the chambers were left in place for more than 1 week. This, of course, limited diffusion and impaired the exchange of gasses, nutrients, and waste products essential for the life of the contained cells. As a result, an arbitrary time limit of roughly 2 weeks was imposed on chamber experiments.

Within a few hours of implantation chambers began to fill with ascitic fluid. Fibrous and reticular elements along with many round cells were found to adhere to the floor and roof of the chamber, but large numbers of lymphocytes, macrophages, and immature cell forms floated free in the accumulating chamber fluid. As a result, a mince of cells placed in the chamber became a cell suspension within 24 hours and could be conveniently aspirated with a tuberculin syringe and needle introduced through either floor or roof.

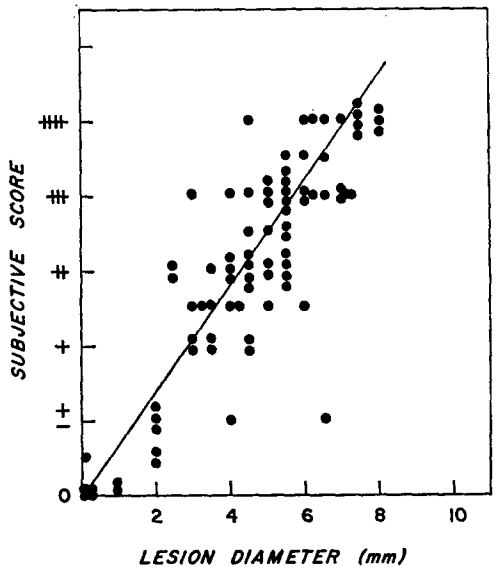
At varying periods of time up to 11 days, the "carrier" rabbits were sacrificed by an overdose of nembutal, and the chambers recovered and transferred to chilled, heparinized (liquemin, 0.05 mg/cc) diluent. Chamber contents were aspirated, diluted in heparinized diluent, and viable cell counts made using a 0.1 per cent solution of trypan blue as the pipette diluent. The cells were then centrifuged at 600 to 800 RPM for 5 minutes at 10°C and suspended in an appropriate volume for injection. A volume of 0.1 ml was injected intradermally in the flank of the specific New Zealand ear skin donor.

Immunization of Lymph Nodes In Vivo.—In many experiments "*in vivo* controls" were run in parallel with the chambers. While three of an animals' lymph nodes were used in chambers, a fourth was left *in situ* and sensitized by subcutaneous implants of the same New Zealand ear skin used to sensitize the lymphoid cells in the chambers. In our hands subcutaneous implants of skin in areas of node drainage led to as good immunization of recipient animals as orthotopic skin grafts and were much more convenient technically. Retroscapular nodes so sensitized were recovered at the same time as the corresponding set of chambers and were minced in chilled diluent to make a suspension. This in turn was counted, centrifuged, resuspended, and injected intradermally in the specific antigen donor exactly as were the cells, recovered from Millipore chambers.

The "Transfer Reaction".—The "transfer reaction" consists of a raised, erythematous indurated lesion, which appears 12 to 18 hours after the injection of sensitized cells and reaches a peak at 48 to 72 hours. Brent *et al.* (14) found that 4 to 10 million lymph node cells were required to give reliable reactions in guinea pigs. With the New Zealand-Dutch rabbit combination, 1 to 2 million cells have been consistently sufficient to produce intense lesions. Thus, by using rabbits we could improve the sensitivity of the system and reduce the non-specific artefact occurring when large numbers of cells are injected intradermally.

Quantitating the "transfer reaction" presents something of a problem. When soluble, diffusible antigens such as tuberculoprotein are injected intradermally in an actively or pas-

sively immunized host, the diameter of the resulting skin lesion is an adequate measure of the intensity of the reaction. Since soluble antigen diffuses widely, these reactions may achieve diameters of 2 to 3 cm. With the "transfer reaction," and indeed with other forms of local passive cutaneous transfer, scoring is more difficult. The injected cells do not diffuse widely through the skin of the host. Therefore, strong and weak reactions may differ only slightly in diameter; yet they may differ considerably with regard to elevation, color, and degree of induration. Following Brent *et al.* (14) we have graded "transfer reactions" arbitrarily, using a scale of 0 to ++++, taking into consideration these other factors as well as diameter. Text-fig. 2 provides a chart for translating the subjective scoring units used throughout this paper

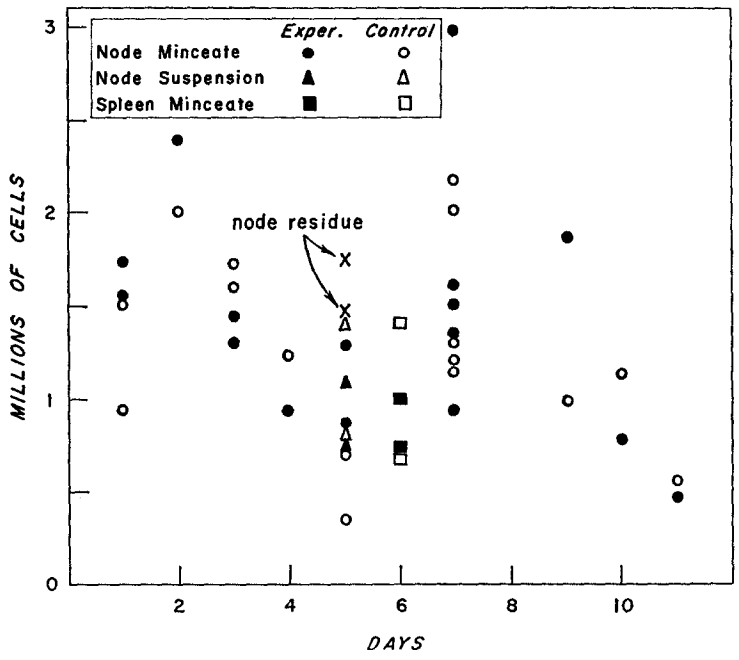


TEXT-FIG. 2. Transfer reactions: the relation of subjective score to diameter of individual lesions.

into diameters of reactions in millimeters. It is clear that these parameters have a first order relationship, but that relatively large increases in the subjective score are accompanied by much smaller changes in diameter. Thus, a dark red, raised and indurated reaction scored as ++++ may be only 4 mm in diameter larger than a relatively flat, pink lesion scored as +. Nevertheless, these reactions are obvious both to the eye and to palpation, and scoring them on an arbitrary scale is simple, accurate, and reproducible.

The histological character of the "transfer reaction" in the rabbit will be described fully in a subsequent publication. In general, control (non-sensitized) cells injected intradermally were readily identifiable over several days as isolated nests of cells in the deeper dermis and between the follicles. These tended to increase in size and additional cells were observed by 2 to 3 days about follicular vessels and about large venous and lymphatic channels in the deep dermis and underlying tissue. The principal cellular elements were lymphocytes, small mononuclear cells of indeterminate character, and histiocytes. In some specimens many eosinophils were observed, and cells of the plasma cell series began to appear on the 3rd day. When specific sensitized cells were injected intradermally the nests of injected cells increased rapidly in size from

day 1, reaching a maximum at 3 days. At this time, while they had not increased in number, they became so large as to coalesce and to form an almost continuous mass of large, pale histiocytes, with smaller numbers of other cell types extending throughout the dermis of the injection zone, about the follicles, and frequently to the surface epidermis. Many eosinophils were present from the 1st day, foci of necrosis were sometimes observed, and a vigorous plasma cell response, largely perivascular, was in progress by the 3rd day. The extent of the positive reaction agreed closely with the arbitrary score described above, and bore a clear relation to the number of injected cells.



TEXT-FIG. 3. Cell survival in Millipore chambers as a function of time. Each point represents the average cell survival per chamber in a single experiment.

RESULTS

Cell Survival in Chambers.—The data obtained from viable cell counts of chamber aspirates are presented in Text-fig. 3. Only data from chambers containing lymph node mince are available over the full 11 day time span. More than 95 per cent of cells recovered from chambers at various intervals were living as measured by the trypan blue method. In contrast, cell suspensions prepared directly from lymph nodes were only 70 to 80 per cent viable.

During the first 24 hours, the number of living cells obtained from chambers fell sharply to 10 to 20 per cent of the starting figure. Thereafter, the chambers became stabilized and counts of 0.5 to 3.0 million viable cells per chamber were

obtained for the duration of the experiment.[1] Cell survival in both experimental and control chambers appeared to follow a biphasic pattern, with slight peaks on days 1 to 3 and 7 to 9. Since experimental and control points parallel each other closely, it is unlikely that the variations in numbers of cells recovered on different days reflects any immunological reactivity on the part of the contained lymphoid cells. A biphasic survival has not been reported by other workers who have cultured lymphoid cell suspensions.

Lymph node cell suspensions and spleen minces gave viable cell counts on days 5 and 6 comparable to those obtained with lymph node minces (Text-fig. 3). Lymph node residue, which consisted of the material left behind after the node had been minced and washed 4 to 6 times, apparently gave as good suspensions at 5 days as the corresponding whole mince.

The counts shown in Text-fig. 3 represent free cells floating in the chamber fluid. Large numbers of additional cells, not recovered by routine washing, were attached to the inner surfaces of the filters. Incubation of chambers for 30 minutes in 0.5 per cent trypsin at 37°C yielded a further number of cells equal to or greater than that collected from the initial aspiration. Such cells did not, however, produce "transfer reactions" when injected into the specific antigen donor. It remains possible that a milder form of treatment might lead to the recovery of functionally intact cells from the chamber walls.

Bacteriological analyses done at random showed the chambers used in the present experiments to be sterile. Yet the shaved and alcohol-washed skin placed in most chambers was clean but not sterile. It is likely, therefore, that the lymphoid cells present in each chamber were able to cope with a small inoculum of bacteria. In experiments not reported here, non-sterilized chambers were found on recovery to be overrun by Gram-positive cocci or Gram-negative rods. In such chambers lymphoid cells survival was severely reduced, and often no viable cells could be recovered.

Morphologic Character of the Transferred Cells.—Giemsa-stained smears were made of the contents of chambers filled with lymph node mince and recovered at various intervals. Lymphocytes and young forms in the lymphocyte series were the predominant cell types in all chambers, giving a biphasic frequency curve roughly parallel to the total cell survival curve (see Text-fig. 3). 1 to 3 per cent of the initial cell populations of both experimental and control chambers were monocytes. By day 3 or 4 typical large macrophages with abundant foamy cytoplasm appeared and these made up 10 to 30 per cent of the total cell population after day 4. Also, from day 4 on, the number of such cells was consistently greater in experimental than in control smears. Large,

[1] Holub (11), culturing suspensions of rabbit lymphoid tissues in chambers of the same approximate dimensions but with different filters, reports survival of 10 to 15 million cells per chamber. He used collodion filters, 60 μ thick and with a pore size ranging from 0.25 to 0.6 μ (standard error of the pore size not given).

elongated, non-basophilic cells resembling reticulum cells were occasionally seen in the first 4 days of culture. Blasts made up roughly 1 per cent of the cell population at all time intervals. Plasma cells and type II lymphocytes were rare at all times. Smears of cells removed from chamber walls with trypsin after 11 days of culture showed a somewhat higher per cent of macrophages and unidentifiable forms and a lower per cent of lymphocytes. None of these cell types appeared in greater frequency in experimental as compared with control chambers.

TABLE I

Data from Single Experiment Comparing "Transfer Reactions" Produced in New Zealand Rabbit Skin by Dutch Rabbit Lymph Node Cells After Contact with Donor Ear Skin in Millipore Chambers and in the Intact Animal

Source of cells	Experimental (E)* or control (C)‡	Cells transferred $\times 10^6$	Reactions at		
			24 hrs.	48 hrs.	72 hrs.
Chambers, "carrier"1	E	1.1	++	+++	++ to +++
	C	1.2	0	0	0
Chambers, "carrier"2	E	2.1	++ to +++	++++	+++ to ++++
	C	2.1	0 to ±	±	0 to ±
Pooled chamber supernates	E	0	0	0	0
	C	0	0	0	0
In vivo lymph node cells§	E	1.8	+ to ++	++	++

* E: Dutch rabbit cells exposed to New Zealand rabbit skin for 10 days.

‡ C: Similar cells exposed to autologous Dutch rabbit skin for 10 days.

§ Cells of Dutch recipient's lymph node draining site of implantation of New Zealand rabbit skin grafts.

Immunological Activity of Cells Recovered from Chambers.—Normal Dutch lymphoid tissues, exposed to New Zealand ear skin or epidermal cell suspensions (one experiment) for suitable periods of time in Millipore chambers, produced typical "transfer reactions" upon intradermal injection in the specific New Zealand ear donor. A variety of Dutch control lymphoid cells failed to do so. The protocol of a typical experiment is shown in Table I. Sites injected with experimental cells showed clear positive reactions by 24 hours. These became maximal at 48 hours and were little changed at 72 hours. Control cells and 0.15 ml of supernatant fluid from experimental and control chambers failed to give significant reactions at any time.

A summary of the 48 hour readings from all our experiments is presented

TABLE II

48 Hour Transfer Reactions Obtained with Cells Immunized in Chambers and In Vivo for Different Lengths of Time

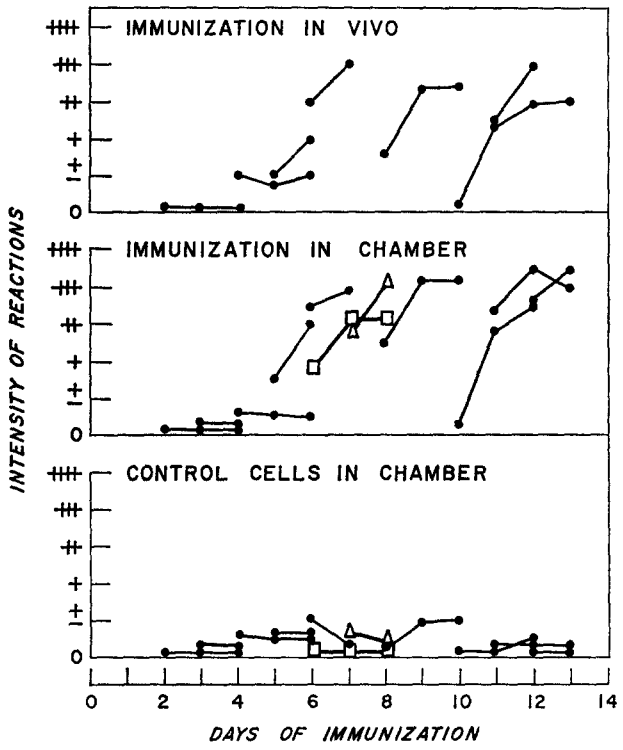
Days in chamber	Type of cell cultured	Number of cells × 10^6		Reactions at 48 hours	
		Test*	Control	Test*	Control
1	Lymph node mince	2.3, 2.3, 2.4, (2.5)	1.3, 2.5	0, 0, 0, (0)	0, 0
1	" " "	2.0, 1.0, (1.9)	2.0, 1.0	0, 0, (0)	0, 0
2	Lymph node mince	3.1, 3.1, 3.1	3.0, 3.0	0 to ±, 0 to ±, 0 to ±	0 to ±, 0 to ±
3	Lymph node mince	3.4, 2.1, (3.0)	3.4, 3.0	±, 0, (±)	±, 0
3	" " "	2.6, 2.6, (2.5)	2.6, 2.6	0 to ±, 0 to ±, (0 to ±)	0 to ±, 0 to ±
4	Lymph node mince	4.3, (3.0)	3.9	++, (+)	0 to ±
5	Lymph node mince	2.4, 1.0	1.3	+++, +++	0
5	" " "	3.5, 1.7, (2.5)	2.8	++++, + to ++, (+++)	0 to ±
5	Lymph node cell suspension	3.2	2.5	++ to +++	0
5	" " " "	5.1	4.5	0	0
5	" " " "	2.3	2.2, 2.2	+ to ++	0, 0 to ±
5	Lymph node residue	3.4	—	++ to +++	—
5	" " "	2.4	—	0	—
5	" " "	2.7	—	+ to ++	—
6	Spleen mince	4.1	4.1	++ to +++	0 to ±
6	" "	3.6	2.0	++++	0
7	Lymph node mince	4.0, (5.0)	1.3	++++, (++ to +++)	0
7	" " "	6.0, 2.5, 4.7‡	6.2, 3.4	+++, ++++, ++++‡ (++)	+, + to ++
7	Lymph node mince	3.3, 2.7, (10.0)	2.2, 2.2	0, 0, (0)	0, 0
7	" " "	6.4, 2.4, (2.5)	4.3	++ to +++, + to ++, (++ to +++)	0
7	" " "	2.7	2.3	++++	0
9	Lymph node mince	3.7, 3.8, (5.0)	3.9	++ to +++, +, (+ to ++)	0
10	Lymph node mince	1.1, 2.1, (1.8)	1.2, 1.2, 2.1	+++, ++++, (++)	0, 0, ±
11	Lymph node mince	1.4	1.1	+++ to ++++	0

* Data for lymph node cells sensitized *in vivo* given in parentheses.

‡ Antigen used in this experiment was epidermal cell suspension (1.2 × 10^6 epidermal cells per chamber).

in Table II. Text-fig. 4 is a plot of the 24, 48, and 72 hour readings with cells taken after varying immunization periods; it compares the results obtained with lymphoid cells sensitized in chambers, lymphoid cells sensitized *in vivo*, and control chamber cells. Lymph node mince in chambers and lymph node

cells *in vivo*, exposed to antigen for less than 4 days, failed to produce "transfer reactions" when injected intradermally in the specific New Zealand antigen donors. Cells exposed to homografts for 4 days in chambers or *in vivo* were capable of initiating weak "transfer reactions." Cells exposed to antigen for



TEXT-FIG. 4. Transfer reactions as a function of the number of days of immunization (in chambers or *in vivo*). The data were pooled from experiments carried out with each immunization time. The two (or three) connected points represent the average 24, 48 (and 72) hour readings for all experiments with immunization for this length of time. ●, Lymph node mince immunized in chambers or draining node immunized *in vivo*; △, Spleen mince immunized in chamber; □, Lymph node suspension immunized in chamber.

longer periods of time in chambers or in the intact animal, elicited strong "transfer reactions" in nine out of ten experiments. Reactions produced by cells sensitized in chambers had the same appearance, intensity, and time course as reactions produced by cells immunized in the intact animal.

In most experiments with a sufficient period of immunization, reactions had begun by 24 hours. In one experiment (9 days immunization) cells recovered from experimental chambers and from the intact Dutch rabbit sen-

sitized in parallel failed to give reactions at 24 hours but gave strong reactions at 48 and 72 hours. In another experiment (7 days' immunization) cells obtained from chambers and from the intact animal failed to give reactions at any time. When the donor of lymph node cells was pregnant (one experiment), neither the cells recovered from chambers nor those from the intact, pregnant animal were able to produce a "transfer reaction." These aberrant results illustrate at once the advantage of *in vivo* controls for chamber experiments and the strict parallelism of immunological activity which exists between cells sensitized *in vivo* and in chambers.

Spleen mince cultured in chambers in the presence of antigen was found to be as effective as lymph node mince in producing "transfer reactions." It was not possible to run *in vivo* controls on these experiments because entire spleens were required for the chamber minces. Splenic cells of animals sensitized with subcutaneous implants of ear skin failed to give "transfer reactions" on injection into the antigen donor. Suspensions of lymph node cells gave definite "transfer reactions" after exposure to ear skin antigen in chambers, but these reactions were less intense than those produced by equal numbers of node or spleen mince cells. The residues from these cell suspensions, when cultured with New Zealand ear skin, gave rise to suspensions of cells capable of inducing definite "transfer reactions."

Dutch lymph node or spleen mince, or lymph node suspension, cultured alone or with autologous Dutch ear skin, when injected into New Zealand rabbits gave reactions scored as 0 or 0 to ±; a single control lesion was rated as + to ++. The injection of cells immunized against skin of a specific New Zealand rabbit into an unrelated New Zealand animal or the Dutch lymph node donor produced no reaction. Finally, immunized cells killed by heat (48°C for 20 minutes) were unable to produce reactions in the New Zealand ear skin donor.

Character of "Transfer Reactions" Produced by Cells Immunized in Chambers.— Experimental chamber cells, injected intradermally in the specific New Zealand skin donor, gave "transfer reactions" histologically indistinguishable from those produced by cells sensitized *in vivo* (see Figs. 1 to 3). The lesions consisted essentially of discrete islands of cells. By 24 and 48 hours many more cells were present in the lesions than were injected. These were chiefly large histiocytes whereas the injected cells were predominantly lymphocytes. Control cells, whether obtained from chambers or from non-sensitized Dutch rabbits, gave a much milder histologic picture consisting of small, scattered clumps of cells.

DISCUSSION

Our data show clearly that normal Dutch lymphoid cells, exposed to New Zealand skin homografts in chambers, are capable of producing "delayed" skin lesions in the specific New Zealand ear skin donor. That these are typical

"transfer reactions" with all the properties of immunological phenomena is attested by the following points. First, experimental chamber cells produced reactions with the same appearance, intensity, time course, and histological character as cells sensitized in the intact animal while control cells cultured in parallel with the experimental cells failed to provoke reactions. Second, an immunizing or latent period was required before cells exposed to antigen were capable of producing reactions. Animals sensitized with a variety of protein antigens are capable of an immune response no sooner than the 4th or 5th day (16). Homografts first show signs of rejection on the 5th day (17), and cells to be used for systemic passive transfers of tumor immunity must be sensitized for at least 4 days (18). Brent (14) found that "transfer reactions" could not be produced by cells sensitized for less than 4 days. That cells in Millipore chambers showed no immunological activity after exposure to antigen for 3 days, slight activity after 4 days of exposure, and strong activity thereafter suggests the induction of an immune state. Thirdly, a strict parallelism exists between the immunological activity of cells sensitized in chambers and cells sensitized in the intact animal. Thus, when the immunizing period was insufficient or the Dutch lymphoid donor was pregnant, neither set of cells gave reactions. In one aberrant experiment, neither set of cells gave reactions at 24 hours but both gave strong reactions at 48 and 72 hours. Fourth, the specificity of the reactions must be stressed. Cells sensitized in chambers or *in vivo* provoked no reaction when injected into the animal providing the lymphoid tissue. Yet, the same cells gave strong reactions when injected into the specific antigen donor. Finally, only the living cells recovered from chambers, not disrupted cells or chamber fluid, could provoke reactions. This is consistent with Brent's (14) finding in intact animals that only viable cells, not heat-killed cells or serum, are able to elicit reactions. It is a fundamental property (19) of other types of delayed hypersensitive reactions, among them bacterial allergy, contact allergy, and certain types of homograft rejection, that passive transfer can only be accomplished with viable, sensitized cells.

Thus, there can be no doubt that lymphoid cells exposed to homograft antigen in Millipore chambers undergo primary sensitization and that Millipore chambers afford a convenient tool for studying the activity of immunologically competent cells. Holub's (11) observations on primary antibody formation in chambers are hereby confirmed in a system involving sensitization of the delayed type. There remains, nevertheless, a discrepancy between the lymphoid cell survival in chambers reported by Holub and that obtained in the present experiments. After inoculating his chambers with 31 to 93 million cells in suspension, he found cell survival at 8 days to be in the range of 10 to 15 million cells per chamber. In our study, whether large inocula comparable to those employed by Holub or smaller numbers of cells were employed, the viable cell count generally ranged from 1 to 3 million cells per chamber. In the present

experiments, therefore, 10 to 20 million cells per chamber were used, whether in suspension or as mince. In Holub's study plasma cells and type II lymphocytes were more prominent in experimental than in control chambers. In our experiments typical macrophages were prominent during the period when reactive cells were present in the chambers and less so in chambers containing control cells.

The latency period required for sensitization of lymphoid cells to protein and homograft antigen also warrants comment. Dienes and Mallory (20) postulated that delayed hypersensitivity might be a first or primitive stage in the development of cells capable of forming antibody. If this is true, such delayed hypersensitivity should appear before antibody is formed. The comparison of our results with those of Holub speaks against this hypothesis. Using the "transfer reaction," a sensitive system requiring no more than 2 million viable cells, to measure the presence of delayed hypersensitivity, we could detect no immunity before the 4th day of immunization in chambers. Using a similar culture system, Holub was able to detect antibody against protein and bacterial antigens after 3 to 4 days of sensitization. Thus, it would seem that antibody formation and delayed hypersensitivity develop at similar rates in membrane chambers rather than in sequence.

The potential usefulness of Millipore chambers for analyzing the process of immunization and the capacities of different immunologically competent cells need hardly be emphasized. In the present study it was established that minces of spleen, exposed to antigen in chambers, gave excellent "transfer reactions" whereas splenic cells taken from sensitized intact animals failed to do so. It is probable that antigen from skin slices planted orthotopically or subcutaneously, while sensitizing the draining lymph nodes, fails to reach the spleen. By crude fractionation of lymph nodes a cell suspension and a residue were obtained both of which were successfully sensitized on exposure to antigen. This confirms the finding of Holub that suspensions of lymph node cells are capable of initiating immunological activity and shows clearly that lymphoid cells are able to undergo primary delayed sensitization independent of the architectural relationships of the intact lymph node.

SUMMARY

Normal Dutch rabbit lymph node and spleen minces, lymph node cell suspensions, and residues from lymph node cell suspensions were cultured in Millipore chambers with slices of autologous or homologous (New Zealand) ear skin for varying time intervals. Lymphoid cells exposed to New Zealand ear skin for more than 4 days were found capable of producing typical "transfer reactions" in the specific New Zealand ear skin donor, similar in every way to reactions produced by cells from lymph nodes sensitized in the intact Dutch animal. Heat-killed cells and cells exposed to New Zealand ear skin for less

than 4 days (in chambers or in the intact animal) or to Dutch ear skin for any period of time were incapable of eliciting such reactions. It is concluded that normal lymphoid tissues undergo primary sensitization when exposed to homografts in Millipore chambers for suitable periods of time.

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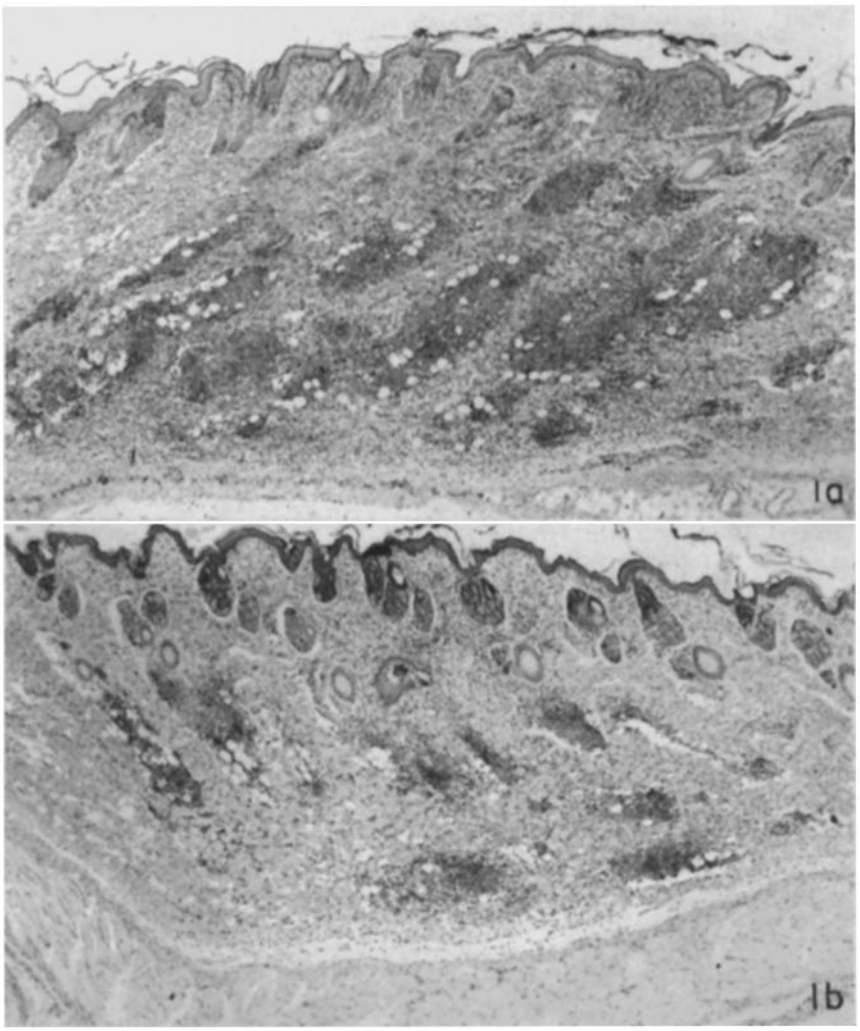
EXPLANATION OF PLATES

PLATE 1

FIG. 1. Transfer reactions (72 hours) produced by a single batch of lymph node mince cells cultured in chambers with homologous (*A*) and autologous (*B*) skin for 7 days. The number of cells injected was 2.5×10^6 (*A*) and 3.4×10^6 (*B*); and the reactions were scored grossly as +++ and + to ++. Hematoxylin and eosin, × 35.

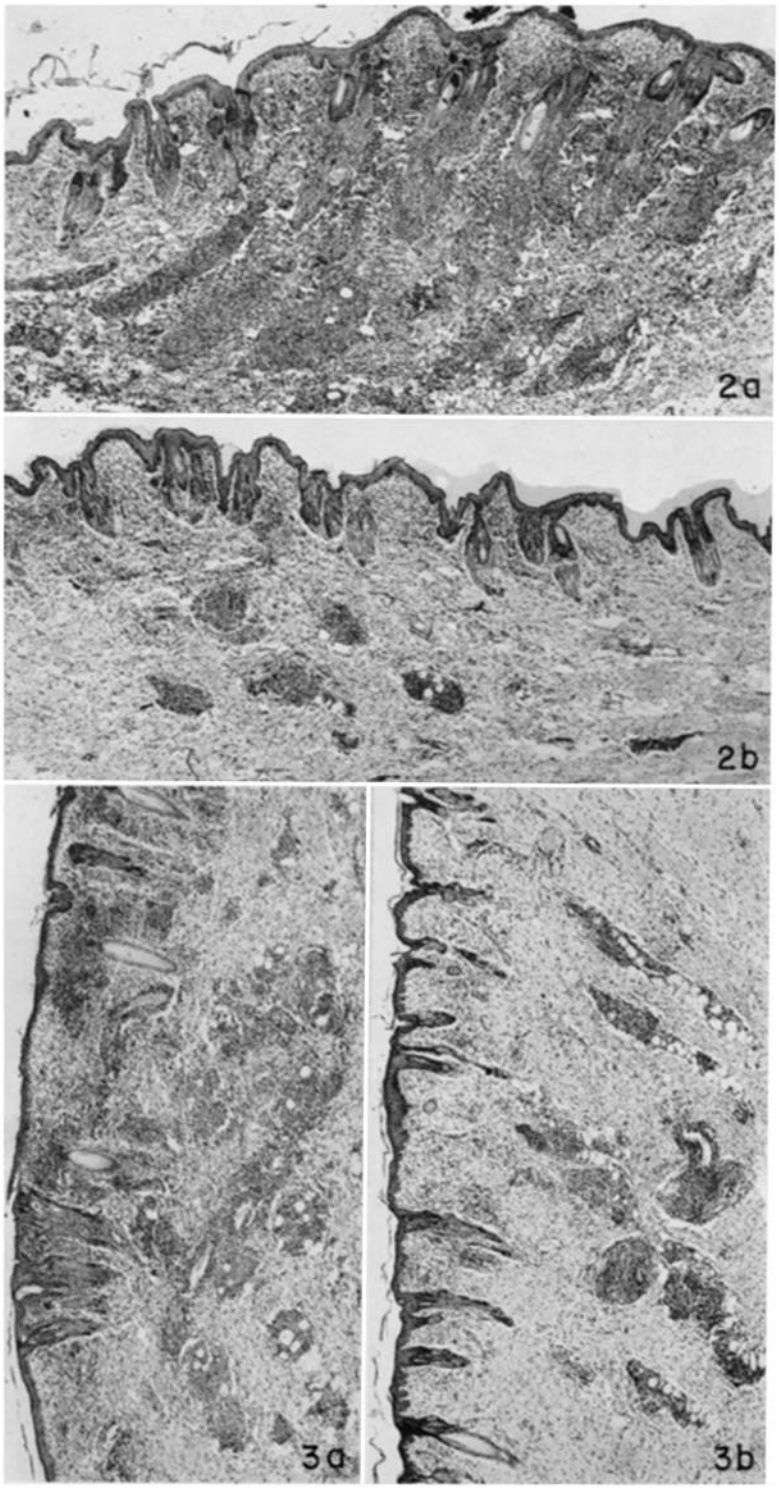


(Dvorak and Waksman: Lymph node cells)

PLATE 2

FIG. 2. *A*. Transfer reaction (48 hours) produced by 3.0×10^6 cells of lymph node draining site of graft implantation, after 10 day immunization period *in vivo*. *B*. Reaction produced in same test skin by 3.0×10^6 normal cells from a non-immunized rabbit. Gross scores were $+++$ (*A*) and $\pm$ (*B*). Hematoxylin and eosin, $\times$ 35.

FIG. 3. Transfer reactions (72 hours) produced by a lymph node cell suspension exposed in chambers to homologous (*A*) and autologous (*B*) skin for 5 days. Cell doses were both 2.2×10^6; and reactions were scored as $+$ to $++$ and 0. Hematoxylin and eosin, $\times$ 35.



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