THE NATURE AND THE SPECIFICITY OF MONONUCLEAR CELLS IN EXPERIMENTAL AUTOIMMUNE INFLAMMATIONS AND THE MECHANISMS LEADING TO THEIR ACCUMULATION*

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The relative importance of sensitized cells and autoantibodies in the pathogenesis of most forms of experimental autoimmune diseases has not been fully clarified. A widely held view, however, is that in models which are characterized by mononuclear cell infiltration the tissue damage is mediated directly or indirectly by sensitized cells (1–3) and that the inflammation is essentially a delayed hypersensitivity reaction. The most compelling argument in favor of this interpretation and against a major role of humoral antibodies is that these diseases can be transferred with lymphoid cells (4–6) but generally not with serum.

Although there may be several mechanisms by which the transferred sensitized cells evoke inflammation, the simplest explanation for the initiation of the reaction would appear to be that some specifically sensitized cells localize in the target tissue and come in contact with the appropriate antigen. If this is so, one would expect to find specifically sensitized cells in the lesion. This problem has been studied in delayed sensitivity reactions (to exogenous antigens), principally in transfer studies employing labeled cells traced by radioautography, and on the basis of several studies (7–10) the following conclusions have been reached. The majority of cells are derived from a population of rapidly dividing cells formed in the bone marrow; these cells appear as monocytes in the circulation, which after emigration differentiate into macrophages. In experiments in which delayed sensitivity was transferred with labeled lymph node cells, small but significant numbers (3–10%) of labeled cells were found in the reactions. Although these labeled cells may have included macrophages or monocytes, presumably most of them were lymphocytes, and some of them may have been specifically sensitized cells. However, in most studies, approximately equal numbers of labeled cells

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were found in reactions elicited by the antigen to which the donor had been sensitized and by unrelated antigens; thus, these observations failed to provide evidence for preferential accumulation of specifically sensitized cells.

Similar studies on cellular specificity have not been carried out in autoimmune diseases. The purpose of the present study was to explore the nature and specificity of the mononuclear cells in two passively transferred autoimmune diseases and to study the mechanisms responsible for the accumulation of the cells. Particular attention was placed on distinguishing between lymphocytes and monocytes or macrophages. Two recently described models for cellular transfer of rat experimental allergic adrenalitis and encephalomyelitis were used. It was shown by Levine and Hoenig that the success of passive transfer of these diseases, by means of lymph node cells, is enhanced by burning a small area on the surface of the adrenal gland or brain of the prospective recipient (11). Within 24 hr after transfer of cells from appropriately sensitized donors, a mononuclear cell infiltrate develops on the border of the necrotic area in the homologous tissue but not in other areas of the tissue and not in other tissues. Because of the greater reproducibility of transfer and because of the rapid development of inflammation in the target tissue, these models are ideally suited for studies designed to trace labeled donor cells in the recipient.

Materials and Methods

Animals.—Female Lewis rats (Simonsen Laboratories, Gilroy, Calif.) of an inbred colony were used. For each experiment, the rats were selected at random from a pool of about 100 rats at a time when their body weights were between 220 and 240 g. They were divided into groups of 8–12 per cage and fed food pellets and tap water ad libitum.

Immunization of Donor Rats.—Adrenal-adjuvant and spinal cord-adjuvant emulsions containing 250 ml of tissue antigen per milliliter and saline solution-adjuvant emulsion, all fortified with 2 mg of *Mycobacterium tuberculosis* and 2 mg of *Mycobacterium butyricum*/ml, were made as described in a preceding paper (12). For adrenal-adjuvant emulsions, adrenals from Lewis rats were used. For spinal cord-adjuvant emulsions, guinea pig spinal cords were used in early experiments and Lewis rat spinal cords in later ones. The tissues were used fresh, or stored in sealed containers at −20°C for up to 2 months before use.

The donor rats were injected with 0.06 ml of tissue homogenate-adjuvant emulsion divided among three of the right hind footpads. The footpad injection was immediately followed by an intra- and subcutaneous injection of 0.1 ml of concentrated pertussis vaccine (Parke, Davis & Co., Detroit, Mich.) in the dorsum of the same foot (12). Donors of pertussis-sensitized cells were injected with saline solution–adjuvant emulsion and pertussis vaccine. On the 8th or 9th day, when the donor rats were killed and the lymph nodes harvested, both adrenals and the brain stem were removed and processed histologically. The incidence of adrenalitis in adrenal-sensitized and of encephalomyelitis in spinal cord-sensitized rats was 66% or more in the donor rats. The brain stems of adrenal-sensitized donors and the adrenals of spinal cord-sensitized donors never showed inflammation, indicating topographic specificity in the response to these tissue antigens.

Production of Heat Lesions.—Heat lesions in adrenal and brain were produced in prospective recipients 4 or 5 days before the cell transfer. The procedures were slightly modified from those

cited in reference 11 in that a soldering iron with a pointed tip was used (4035 heating unit, 47½–56½ w, with a ⅛ inch tiplet, tip temperature 800–850°F, Ungar). The exposed left adrenal was burned in one spot for 1 sec and the right cerebral hemisphere through the intact calvarium in one spot for 12 sec.

X-Irradiation of Recipients.—Whole body irradiation of recipients was performed with the rats in Lucite chambers on a rotating board, using a Westinghouse X-ray machine (200 kvp, 25 ma, filters 1.56 mm Cu and 0.54 mm Al, target-source distance (TSD) 50 cm, the dose rate being 23.5 R/min, air or TSD 80 cm, the dose rate being 13.25 R/min, air). The rats were exposed to 400 or 800 R. For lead shielding of the tibial bone marrow, 3 mm lead plates (excluding more than 99.5% of the delivered dose of irradiation) were wrapped around both legs from the middle of the thighs to the toes.

Donor Lymph Node Cell Suspensions.—Donors of lymph node cells were killed with an overdose of ether and their right popliteal, subiliac, and lumbar lymph nodes immediately removed. The nodes from a group of donors were pooled in chilled tissue culture medium 199 (Grand Island Biological Co., Grand Island, N. Y.) and processed separately. The cell suspensions were made by mincing the nodes with scissors and forceps and gently straining the tissue through a No. 100 stainless steel mesh together with fresh medium 199; cell counts were made on the crude suspensions. The cells were washed in medium 199 by centrifugation at 60 × *g* for 6 min.

In Vitro Labeling of Donor Lymph Node Cells.—The suspensions containing cells to be labeled were adjusted to approximately 100×10^6 cells/ml. Normal Lewis rat serum was added to bring its concentration to 10% and the cells incubated with the tritium-tagged nucleoside for 40–60 min at 37°C on a shaking water bath. ^{3}H-thymidine–labeling was carried out with 0.1–1.0 μCi of ^{3}H-thymidine (specific activity 5.7 Ci/mM, New England Nuclear Corp., Boston, Mass.) per ml of cell suspension. ^{3}H-adenosine–labeling was performed with 10 μCi of ^{3}H-adenosine (specific activity 5–15 c/mM, New England Nuclear Corp., Boston, Mass.) per ml of suspension. ^{3}H-uridine–labeling was achieved with 10 μCi of ^{3}H-uridine (specific activity 3.9 Ci/mM, New England Nuclear Corp.) per ml of suspension. Suspensions of cells which were to remain unlabeled were incubated without isotope on the same water bath. After incubation, the cells were spun down at 60 *g* for 6 min, the supernatant carefully decanted, and the cells resuspended in fresh medium 199, and were now ready for transfer. Cell counts and trypan blue tests for cell viability were made on samples of the cell suspensions after the in vitro labeling. In all experiments reported here, more than 90% of the donor cells excluded the dye. Radioautographs of smears of ^{3}H-thymidine–incubated donor lymph node cells showed between 2 and 3% labeled cells. Only lymphoblasts and large lymphocytes were labeled and about 50% of these cells were labeled. ^{3}H-adenosine incubation resulted in labeling of all large cells and of about half of the small lymphocytes. Incubation with ^{3}H-uridine resulted in labeling of up to 90% of the cells. All large cells and most of the small lymphocytes were labeled.

In Vivo Labeling of Recipients Cells.—Labeling of cells of prospective recipients was achieved by intraperitoneal injection of 200 μCi of ^{3}H-thymidine spaced in eight equal doses at 8 hr intervals. The last injection was given 2 hr before the cell transfer.

Lymph Node Cell Transfer.—Cells were transferred to recipients with 4 or 5-day old heat lesions, in a volume of 1–2 ml in the lateral tail vein under ether anesthesia. When cells from two groups of donors were transferred to the same recipients, the cells were mixed in the syringe immediately before injection. After most of the transfers involving labeled cells, a fraction of the labeled cell suspension was centrifuged and smears made from the pellet on gelatin-coated slides. After air-drying for 2 min, they were fixed with methanol.

Bone Marrow Cell Suspensions and Transfer.—Normal rats were bled under ether anesthesia and both femoral bones were removed and freed of soft tissues. The marrow cavity was flushed through with 4°C medium 199 and the flakes of bone marrow were broken by repeated washing

up and down in a syringe. The cells were washed once in fresh medium and were then ready for transfer.

Study of Recipients.—The recipients were killed with an overdose of ether 24 or 48 hr after the lymph node cell transfer. Both adrenals and the whole brain were fixed in buffered formalin. Sections were prepared from the heat lesions for histologic and radioautographic processing. Paraffin sections were cut at 4 μ. The tissue sections and smears of donor cells were dipped in NTB2 emulsion (Eastman Kodak, Rochester, N. Y.) and were developed with D-19 developer after 2–9 wk of exposure at 4°C. The sections were stained with hematoxylin–eosin and the smears with Giemsa stain. Counts of the number of labeled cells were made by Dr. Werdelin on coded autoradiographs which had all been exposed for the same period of time. The area to be examined in the section was chosen at low magnification along the border of the heat lesion and then examined under oil immersion. The counting was facilitated by a standard ocular grid and made at ×1000 magnification. All infiltrating mononuclear cells in the field were counted. Cells belonging to the tissue (adrenal parenchymal cells, endothelial cells, ganglion cells, and glia cells) were not counted. Cells of doubtful origin were counted as infiltrating cells. The focus was then moved to the grain layer and the number of labeled cells was counted. After counting of one field an immediate neighboring field was counted next. Cells covered with 4 grains or more were considered labeled. The background was usually around 30 grains/6400 μ^2 in radioautographs exposed for 28 days.

RESULTS

Transfer of Lymph Node Cells from Donors Sensitized to One Tissue Antigen.—Recipients of cells from adrenal-sensitized donors killed 24 hr or more after cell transfer developed inflammation around the adrenal heat lesions, but not around the brain heat lesions, and the result of transfer of cells from spinal cord-sensitized donors was the reverse. The results of three transfer experiments are compiled in Table I.

The histology of the inflammation developing in adrenal or brain heat lesions after transfer of cells labeled in vitro with either ^{3}H-thymidine or ^{3}H-adenosine was found to be the same as that developing after transfer of unlabeled cells, as described by Levine and Hoenig (11). The adrenal heat lesion at 5 days after the burning in animals not receiving cells from appropriately sensitized donors presented as a semicircular area of necrosis on the surface of the gland extending from the surface of the cortex almost to the adrenal medulla. A few macrophages and neutrophils were generally present on the border between viable and necrotic tissue, but almost no lymphocytes were found. By 24 and to an even greater extent at 48 hr after transfer, a dense infiltrate had developed on the border of the heat lesions (Figs. 1 and 2). Lymphocytes, monocytes, and macrophages were present. Some of the mononuclear cells present could not be definitely identified. However, more than half of the cells in fully developed infiltrates were small lymphocytes. The labeled cells usually were between 1.5 and 5% of all the infiltrating cells. They were found randomly among the unlabeled cells of the infiltrates. The majority of labeled cells were small lymphocytes. Of the remaining labeled cells a few could fairly confidently be classified as large lymphocytes or blasts and a few as monocytes or macro-

phages, while a small proportion of the labeled cells could not be classified.

The size and form of the brain heat lesion was similar to that of the adrenal heat lesion. At 5 days in animals not receiving cells from appropriately sensitized donors a small to moderate number of macrophages were present in the necrotic tissue on the border of the heat lesion, but lymphocytes were virtually absent. At 24 and more so at 48 hr after transfer of cells from spinal cord-sensitized donors numerous lymphocytes and macrophages were found in a band along the border of the heat lesion and perivascularly on the border and deeper in the brain parenchyma (Fig. 3).

In Table II is shown the results of an experiment in which labeled cells from spinal cord-sensitized donors were transferred and their numbers in the brain

TABLE I

Inflammation around Adrenal and Brain Heat Lesions in Recipients of Lymph Node Cells from Adrenal-Sensitized Donors and in Recipients of Lymph Node Cells from Spinal Cord-Sensitized Donors

Treatment of donor rats	No. of recipients* with inflammation around	
	Adrenal heat lesion	Brain heat lesion
Adrenal-sensitized	17/17‡	0/17
Spinal cord-sensitized	0/16	16/16

* Compiled from three separate experiments where recipients of adrenal-sensitized cells were inoculated with 178, 250, and 300 $\times$ 10^6 cells, respectively, and recipients of spinal cord-sensitized cells were inoculated with 15, 122, and 200 $\times$ 10^6 cells, respectively. The recipients were killed from 1 to 5 days after the cell transfer.

‡ Numerator, No. of rats with inflammation; denominator, number of rats inoculated with lymph node cells.

heat lesion counted in radioautographs. The suspension of donor cells was divided into two portions; one was labeled with ^{3}H-thymidine and the other with ^{3}H-adenosine. The recipients were killed 24 and 48 hr after transfer. The percentage of labeled cells in the inflammatory infiltrate of the brain heat lesion was the same at 24 and 48 hr in recipients of cells with the same label. The percentage of labeled cells in the infiltrate of recipients of ^{3}H-adenosine–labeled cells (around 3.5) was about twice that in recipients of ^{3}H-thymidine–labeled cells. Smears of the donor cells, however, showed that whereas 50% of the ^{3}H-adenosine–incubated cells were labeled, only 2% of the ^{3}H-thymidine–incubated were. Since approximately the same numbers of cells were transferred to the recipients in each group, it follows that a much higher percentage of cells which incorporate thymidine accumulate in the infiltrate. Similar results were obtained in experiments in which labeled cells from adrenal-sensitized donors were transferred.

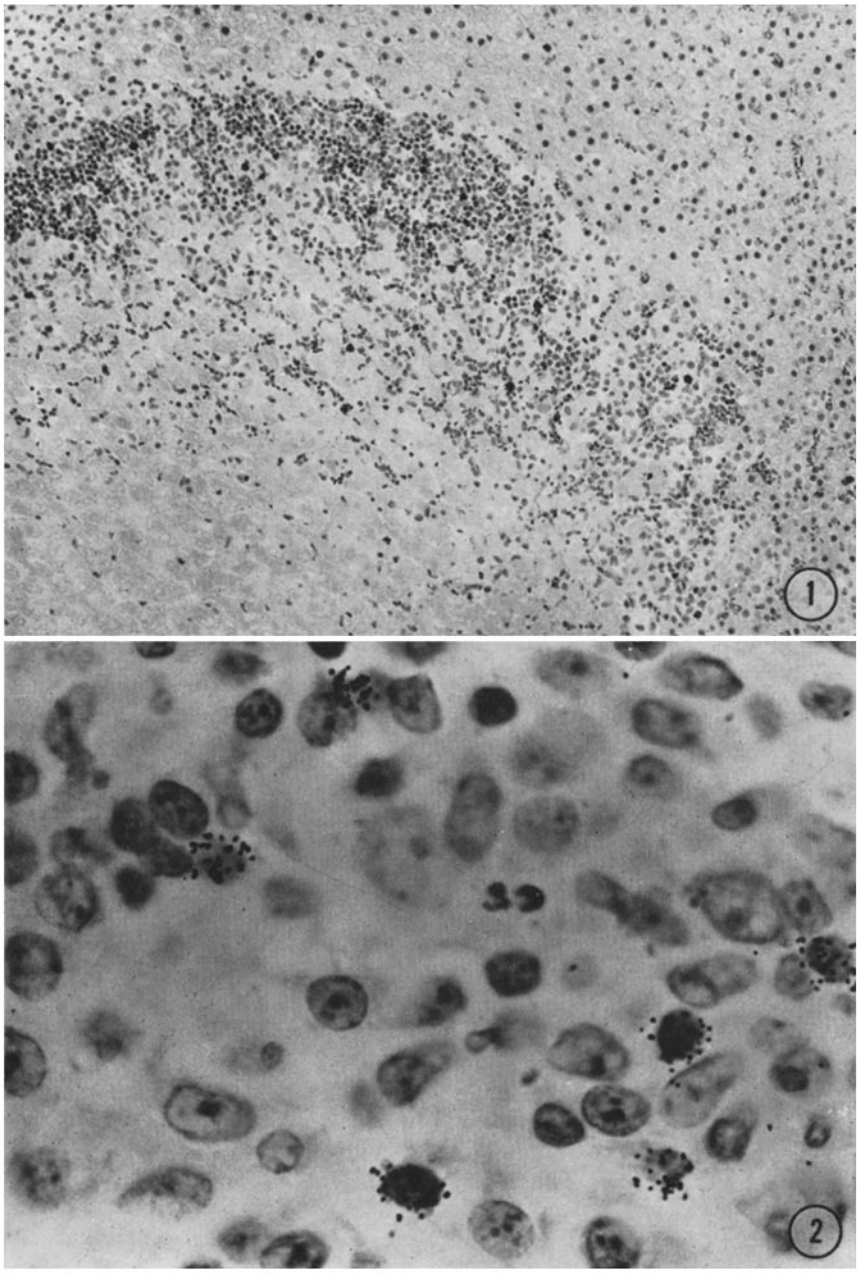

FIG. 1. Adrenal heat lesion in recipient 48 hr after transfer of 178 × 10^6 ^{3}H-adenosine-labeled lymph node cells from adrenal-sensitized donors. Labeled cells are scattered throughout the infiltrate on the border of the necrotic tissue. H & E. × 180. Exposure 28 days.

FIG. 2. Adrenal heat lesion in recipient 24 hr after transfer of 100 × 10^6 ^{3}H-adenosine-labeled lymph node cells from adrenal-sensitized donors plus 100 × 10^6 unlabeled lymph node cells from spinal cord-injected donors. Most of the labeled cells in the infiltrate are clearly lymphocytes. H & E. × 1200. Exposure 6 days.

Accumulation of Host Cells and of Lymph Node Cells from Donors Sensitized to Bordetella pertussis in the Inflammatory Infiltrate around Heat Lesions.— Experiments were designed to assess the extent to which the recipient's own cells accumulate in the inflammation; three prospective recipients with 2-day old heat lesions were injected with ^{3}H-thymidine three times daily for 3 days and then given unlabeled cells from donors sensitized to one tissue antigen. In the one recipient of unlabeled cells from adrenal-sensitized donors, killed

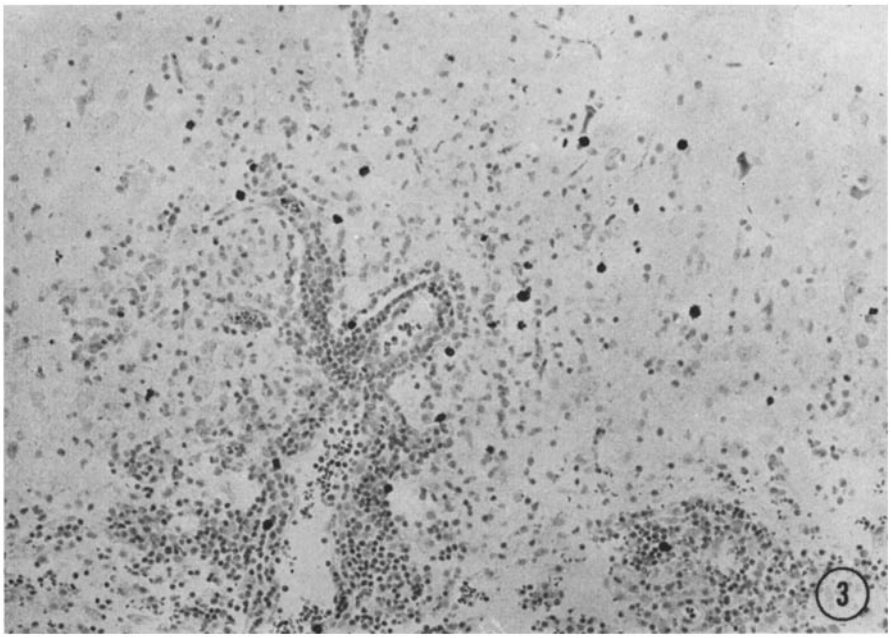

FIG. 3. Brain heat lesion in recipient 24 hr after transfer of 200 × 10^{6} ^{3}H-thymidine-labeled lymph node cells from spinal cord-sensitized donors plus 200 × 10^{6} unlabeled lymph node cells from adrenal-sensitized donors. The labeled cells are distributed randomly in the tissue, whereas most of the infiltrating cells are located perivascularly. H & E. × 180. Exposure 72 days.

48 hr after transfer, 26% of cells in the infiltrate around the adrenal heat lesion were labeled. In the two recipients of unlabeled cells from spinal cord-sensitized donors, killed 38 hr after transfer, 27 and 30% of the cells in the infiltrate around the brain heat lesion were labeled. The labeled cells included both monocyte–macrophages and lymphocytes, the majority being of the former cell type (Fig. 4).

In order to see if lymph node cells from donors sensitized to an unrelated antigen alone would accumulate in the infiltrate, cells from donors sensitized

to *Bordetella pertussis* were labled with ^{3}H-uridine and transferred together with unlabeled cells from donors sensitized to spinal cord or adrenal. Approximately 5% labeled cells were found in the adrenal or brain heat lesion 24 and 48 hr later.

Transfer of Lymph Node Cells from Donors Sensitized to Adrenal Together with Lymph Node Cells from Donors Sensitized to Spinal Cord.—In experiments in which cells from both adrenal-sensitized and spinal cord-sensitized donors were transferred to the same recipient it was found that the inflammatory response developed less often around the heat lesions than in recipients of cells from one type of donor. Only about half of the recipients of donor cells

TABLE II

Percentage of Labeled Mononuclear Cells Around Brain Heat Lesions 24 and 48 Hr after Transfer of Labeled Lymph Node Cells from Spinal Cord-Sensitized Donors

Recipient rat No.	Cells* transferred	Recipient killed hours after transfer	Percentage of labeled cells in radioautographs‡ of the brain heat lesion
2944	Labeled in vitro with	24	1.9
2945	^{3}H-thymidine§	24	1.6
2946		48	1.5
2947		48	1.9
2952	Labeled in vitro with	24	5.0
2953	^{3}H-adenosine‖	24	2.2
2954		48	3.9
2955		48	4.2

* All recipients received 100×10^6 cells.

‡ 1000 mononuclear cells were counted in radioautographs exposed for 35 days.

§ 2% of the donor cells were labeled.

‖ 50% of the donor cells were labeled.

developed inflammation around both heat lesions, the remaining showing inflammation around only one of them.

The result of an experiment in which cells from donors sensitized to adrenal and donors sensitized to spinal cord were mixed and transferred, with those of one type labeled with ^{3}H-thymidine and those of the other not labeled, is shown in Table III. It is evident that the labeled cells (between 1 and 3%) whether from adrenal-sensitized or spinal cord-sensitized donors, were present in the same percentage in the adrenal and brain heat lesions.

Similar transfer experiments in which one type of donor cells was labeled with ^{3}H-adenosine and the other unlabeled also revealed equal numbers (about 5%) of labeled cells around either heat lesion. Recipients examined at 48 or 66 hr after cell transfer showed the same distribution of the labeled cells.

In order to see whether elimination of the recipient's own mononuclear cells

before transfer of cells from tissue-sensitized donors would permit detection of preferential accumulation of labeled donor cells in the appropriate site the following experiment was performed. Rats with 4-day old heat lesions were exposed to 400 R X-irradiation. 1 day later they were injected with lymph node cells from adrenal-sensitized donors and from spinal cord-sensitized donors,

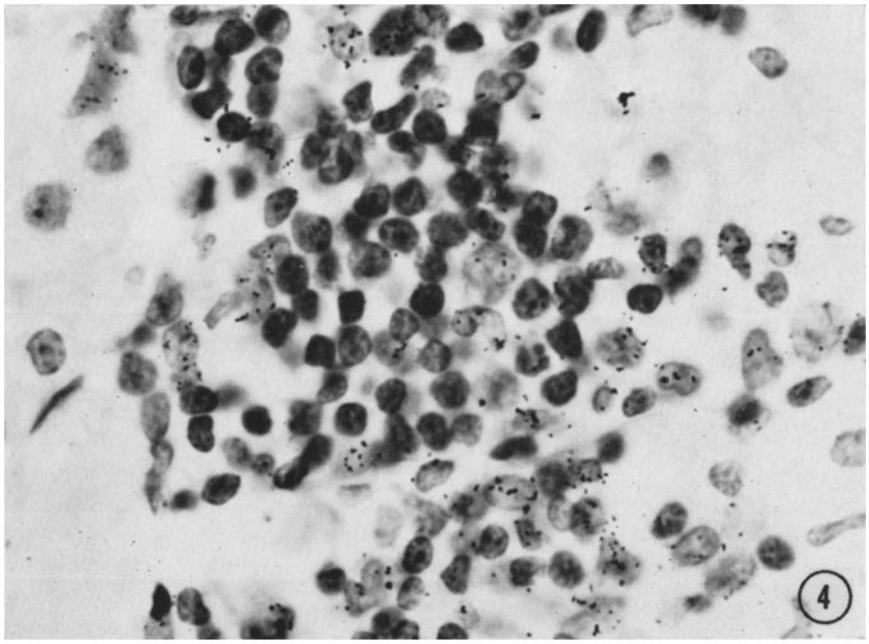

FIG. 4. Adrenal heat lesion 48 hr after transfer of unlabeled lymph node cells from adrenal-sensitized donors. The recipient had been injected with ^{3}H-thymidine during the 3 days preceding the transfer. Most of the labeled host cells are monocytes or macrophages, although some are lymphocytes. H & E. × 1000. Exposure 6 days.

the cells of one type having been labeled with ^{3}H-thymidine. They were sacrificed 2 days later.

The elimination of the recipients own blood mononuclear cells was almost complete 24 hr after X-irradiation by which time the blood count had fallen below 10% of the preirradiation count (Fig. 5). Transfer of donor lymph node cells at this time momentarily elevated the blood mononuclear count, which then slowly declined during the subsequent 48 hr. Only a few small foci of mononuclear cells were found around the adrenal and brain heat lesions, but labeled cells were present in both in a higher percentage than in nonirradiated

TABLE III

Percentage of Labeled Mononuclear Cells around Adrenal and Brain Heat Lesions 24 Hr after Transfer of an Equal Number of Lymph Node Cells from Adrenal-Sensitized and Spinal Cord-Sensitized Donors, the Cells of One Type Having Been Labeled In Vitro with ^{3}H-Thymidine

Recipient rat No.	Labeled cells* from donor rats sensitized to	Adrenal heat lesion		Brain heat lesion	
		Total count‡	% Labeled	Total count	% Labeled
2441	Adrenal§	14/632	2.2	7/610	1.1
2443		7/508	1.4	5/273	1.8
2444		6/410	1.5	11/557	2.2
2445	Spinal Cord‖	21/911	2.3	13/584	2.2
2446		24/733	3.3	19/1050	1.8
2447		25/1115	2.2	35/1085	3.2
2448		26/741	3.5	12/586	2.0

* All recipients received 200 × 10^6 cells from adrenal-sensitized donors + 200 × 10^6 cells from spinal cord-sensitized donors.

‡ In radioautographs exposed for 72 days.

§ 2.8% of the donor cells were labeled.

‖ 2.4% of the donor cells were labeled.

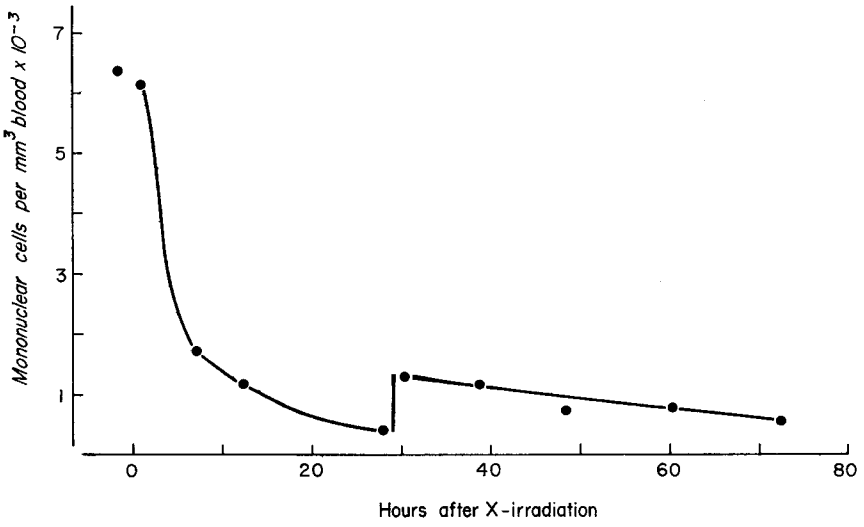

FIG. 5. Number (mean of four) of mononuclear cells/mm^3 of blood in rats given 400 R whole body irradiation and injected intravenously with approximately 338 × 10^6 lymph node cells from donors sensitized to adrenal or spinal cord. Rats were irradiated at the time 0 and the donor cells were injected at the time 29 hr.

recipients. Moreover, the labeled cells were present in the same percentage in the two heat lesions irrespective of which type of donor cells had been labeled (Table IV).

Transfer of Adrenalitis and Encephalomyelitis to X-Irradiated Recipients Harboring Bone Marrow.—In order to determine the role played by cells of bone marrow origin in the development of the inflammation, experiments were carried out in which the myeloid cells of the recipient were eliminated by

TABLE IV

Percentage of Labeled Mononuclear Cells around Adrenal and Brain Heat Lesions of X-Irradiated Recipients 24 Hr after Transfer of an Equal Number of Lymph Node Cells from Adrenal-Sensitized and Spinal Cord-Sensitized Donors, the Cells of One Type Having Been Labeled In Vitro with ^{3}H-Thymidine*

Recipient rat No.	Labeled cells‡ from donor rats sensitized to	Number of labeled cells/total number of cells counted in radioautographs§			
		Adrenal heat lesion Total count	(% Labeled)	Brain heat lesion Total count	(% Labeled)
3090	Adrenal‖	no inflammation		28/387	7.2
3092	Adrenal	17/334	5.1	9/171	5.3
3094	Adrenal	15/181	8.3	25/441	5.7
3094	Adrenal	20/459	4.4	23/290	7.9
3095	Spinal cord¶	31/402	5.2	13/257	5.1
3096	Spinal cord	29/504	5.8	17/350	4.9
3097	Spinal cord	11/197	5.6	22/285	7.7
3098	Spinal cord	14/265	5.3	10/338	5.6

* Whole body X-irradiation with 400 R was administered 24 hr before transfer of lymph node cells.

‡ All recipients received 262 × 10^6 cells from adrenal-sensitized donors + 252 × 10^6 cells from spinal cord-sensitized donors.

§ Radioautographs exposed for 24 days.

‖ 2.2% of the donor cells were labeled.

¶ 1.8% of the donor cells were labeled.

whole body irradiation 5 days before transfer of donor lymph node cells. In some animals receiving irradiation the tibial bone marrow was shielded. Other animals receiving irradiation were injected with normal bone marrow. The design and results of these experiments is shown in Table V. It can be seen that almost all recipients possessing bone marrow cells developed inflammation around the appropriate heat lesion, while none of the other rats did. Histologically, the infiltrate on the border of the inflamed heat lesion of rats possessing bone marrow consisted of the usual mixture of lymphocytes and monocyte–macrophages, although the proportion of the latter was higher than in infiltrates resulting from transfer into intact recipients. In contrast, the border of

heat lesions of rats not possessing bone marrow was almost completely devoid not only of monocyte and macrophages, but of lymphocytes as well.

Accumulation of Cells from Donors Sensitized to Bordetella pertussis in X-Irradiated Infiltrates around Brain Heat Lesions.—The following experiment was designed to see whether the accumulation of lymphocytes in the inflammatory infiltrate depends upon the presence of lymphocytes in the lesion. Rats with 5-day old heat lesions in the brain and adrenal were injected with un-

TABLE V

Inflammation around Adrenal and Brain Heat Lesions after Transfer of Lymph Node Cells from Sensitized Donors to Recipients Irradiated 5 Days Earlier*

Pretreatment‡ of recipients		Donor rats sensitized to	No. of donor lymph node cells injected	No.§ of recipients with inflammation in the target tissue 48 hr after transfer of lymph node cells
Irradiation	Bone marrow cells			
800 R whole body	0	Spinal cord	704×10^6	0/2
800 R with lead shields on legs‖	0	Spinal cord	704×10^6	2/2
800 R whole body	0	Spinal cord	662×10^6	0/2
800 R whole body	303×10^6	Spinal cord	662×10^6	4/4
800 R whole body	0	Adrenal	936×10^6	0/3
800 R whole body	330×10^6¶	Adrenal	936×10^6	2/3
800 R whole body	0	Adrenal	380×10^6	0/1
800 R whole body	270×10^6	Adrenal	380×10^6	3/3

* Compiled from four separate transfer experiments.

‡ The prospective recipients were irradiated in the morning; heat lesions in left adrenal and brain were produced at noon and bone marrow cells from untreated rats were injected in the afternoon on the same day.

§ Numerator, number of recipients with inflammation; denominator, total number of recipients in the group.

‖ The right and left popliteal lymph nodes were removed surgically before the irradiation.

¶ Bone marrow cells from rats injected with saline solution–adjuvant emulsion and pertussis vaccine 9 days before.

labeled cells from spinal cord-sensitized donors. 24 hr later, one group of recipients was given 400 R whole body irradiation; a second was irradiated with 400 R, but with a lead shield on the head; a third group was not irradiated. ^{3}H-uridine–labeled cells from donors sensitized to *B. pertussis* were then transferred to all recipients, which were killed 24 hr after the second transfer. In the brain heat lesions in the irradiated rats the usual number of macrophages was found but very few lymphocytes were present (Fig. 6). The brain heat lesions in the nonirradiated rats contained approximately 10 times as many lymphocytes as did the brain heat lesions of the irradiated group (Fig. 7). Labeled lymphocytes, however, were found with the same frequency per area unit around the lesions of all three groups (Table VI). The experiment demon-

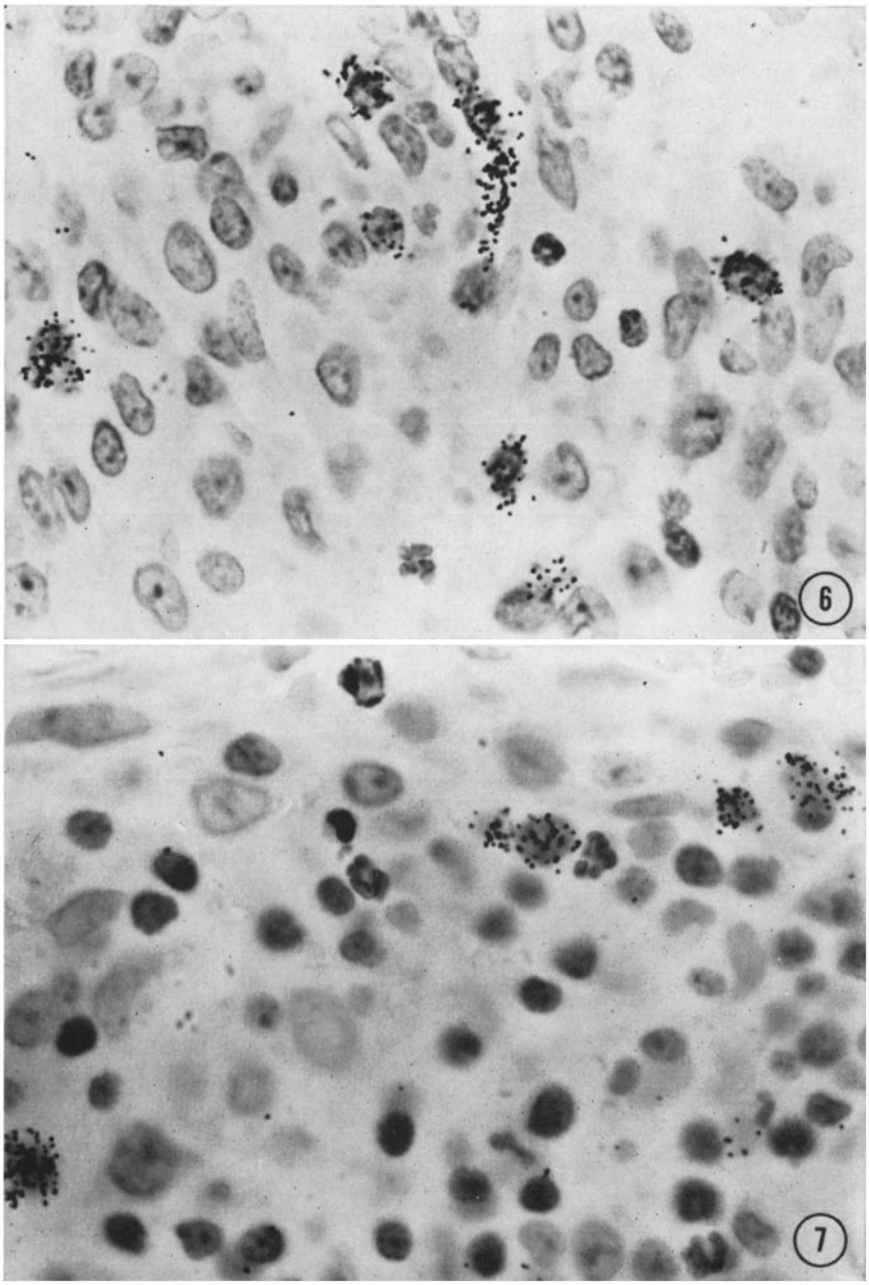

FIG. 6. Brain heat lesion of recipient injected with 212×10^6 unlabeled spinal cord-sensitized lymph node cells, given whole body irradiation 24 hr later and then injected with 200×10^6 ^{3}H-uridine–labeled pertussis-sensitized lymph node cells. The rat was killed 24 hr after the second transfer. The infiltrate contains very few lymphocytes, but most of them are labeled. (Compare with Fig. 7). H & E. × 1000. Exposure 13 days.

strates that the accumulation of lymphocytes in the inflammatory infiltrate continues to occur even though other lymphocytes, including persumably the specifically sensitized cells, are largely eliminated from the lesion.

While irradiation effectively eliminated most of the lymphocytes from the

TABLE VI

Percentage of Labeled Mononuclear Cells around Brain Heat Lesions in Irradiated Recipients of Unlabeled Lymph Node Cells from Spinal Cord-Sensitized Donors Followed 1 Day Later by Transfer of ^{3}H-Uridine–Labeled Lymph Node Cells from Pertussis-Sensitized Donors

Recipient rat No.	Treatment of recipients*		Total No. of lymphocytes/25 fields around the brain heat lesion	No. of labeled lymphocytes/25 fields in radioautographs§ of the brain heat lesion
	Irradiation	^{3}H-uridine labeled cells from pertussis-sensitized donors‡		
3059	400 R with head shielded with lead	166×10^6	121	23
3060	400 R with head shielded with lead	166×10^6	281	24
3061	400 R whole body	85×10^6	81	14
3062	400 R whole body	166×10^6	131	26
3063	400 R whole body	166×10^6	85	27
3064	None	166×10^6	1847	34
3065	None	166×10^6	980	22

* The recipients were irradiated with 400 R 24 hr after transfer of 212×10^6 unlabeled cells from spinal cord-sensitized donors; 3 hr later the labeled cells from pertussis-sensitized donors were transferred. All recipients were killed 24 hr after the second transfer

‡ 65% of the donor cells were labeled.

§ Exposed for 13 days.

heat lesion, it was possible that a few donor lymphocytes remained. To test this, ^{3}H-thymidine–labeled cells from spinal cord-sensitized donors were transferred to four recipients which were given 400 R whole body irradiation 24 hr later and sacrificed 24 hr after that. Only very few labeled cells were found adjacent to the heat lesions, and it was not clear on morphologic grounds how many of them were lymphocytes.

DISCUSSION

In this study of the cellular infiltrate in passively transferred adrenalitis and encephalomyelitis, the prospective recipient rats were prepared by production

FIG. 7. Brain heat lesion of nonirradiated recipient injected with 212×10^6 unlabeled spinal cord-sensitized lymph node cells, and 24 hr later with 200×10^6 ^{3}H-uridine–labeled pertussis-sensitized lymph node cells. The rat was killed 24 hr after the second transfer. The infiltrate contains many more lymphocytes than seen in Fig. 6, but approximately the same number of labeled cells are present. H & E. × 1000. Exposure 13 days.

of a small heat lesion in the adrenal and brain 5 days before transfer of lymph node cells from sensitized donors. At 5 days such lesions showed an area of necrosis, usually bordered by a surprisingly mild inflammatory reaction with a few macrophages and neutrophils, but almost no lymphocytes. Within 24 hr after transfer of lymph node cells, a dense mononuclear cell infiltrate was seen around the lesion in the tissue to which the donor rats had been sensitized, but not around the lesion in the other tissue; lymphocytes were the predominant cell type, although macrophages were also present in large numbers.

In some experiments, transfer was carried out with labeled lymph node cells from donors sensitized to one tissue antigen, the cells having been incubated in vitro with ^{3}H-thymidine or ^{3}H-adenosine. Radioautographs of smears of the donor cells showed that almost all of the labeled cells were lymphocytes or blasts, with very few labeled monocytes or macrophages. Labeled donor cells could be readily detected 24 or 48 hr after transfer in the infiltrate around the heat lesion. Careful study of lightly exposed radioautographs showed that most if not nearly all of the labeled cells were lymphocytes. This appears to be the first demonstration of labeled donor cells in passively transferred autoimmune lesions. The possibility that the infiltration of donor lymphocytes around heat lesions was due to the artificial injection of large numbers of lymph node cells which would not ordinarily enter the circulation is unlikely in view of the findings in another study. There it was shown that accumulation of labeled lymphocytes occurred in actively induced autoimmune adrenalitis 2 days after restricted *in situ* labeling of a lymph node draining the injection site of adrenal tissue.[1] Both observations clearly demonstrate that labeled lymphocytes originating from the lymph nodes draining the injected tissue antigen do indeed accumulate in the site of the autoimmune inflammatory reaction.

It was also observed that donor cells incorporating ^{3}H-thymidine, i.e. proliferating cells, showed a far higher affinity for the heat lesion than ^{3}H-adenosine–labeled cells, which include a large number of long-lived lymphocytes. The propensity of newly formed lymphocytes to accumulate in inflammatory infiltrates has been described in other systems. Thus, Porter and Calne (13) and Gowans et al. (14) have shown that the lymphocytes which invade skin homografts are mainly newly formed cells, and Koster and McGregor (15) have shown that among thoracic duct lymphocytes it is only the newly formed cells, with a short life-span in the blood stream, which accumulate in casein-induced peritoneal exudates. Similarly, Koster et al. (16) have found that it is primarily the recently formed lymphocytes which participate in hypersensitivity reactions directed against bacterial antigens.

[1] Werdelin, O., G. Wick, and R. T. McCluskey. The fate of newly formed lymphocytes migrating from an antigen stimulated lymph node in rats with allergic adrenalitis. Submitted for publication.

On the basis of the percentage of labeled donor cells in the lesions (usually fewer than 10%), it seemed likely that most of the mononuclear cells were of recipient origin. This was shown to be the case; recipients with heat lesions were injected with ^{3}H-thymidine three times daily for 3 days before transfer of unlabeled cells from spinal cord or adrenal-sensitized donors, and at 24 or 48 hr approximately 25–30% of the mononuclear cells bordering the lesions were found to be labeled. Some of these were clearly lymphocytes; however, in contrast to the transfer studies in which labeled donor lymph node cells were used, the majority of labeled cells appeared to be monocytes or macrophages.

The infiltrate developing in the target tissue is thus made up of several components. There is a small but significant number of donor lymph node cells, many of which are newly formed, and a large number of recipient lymphocytes which are also newly formed, and of recipient monocyte–macrophages.

The finding of many lymphocytes of recipient origin in the infiltrate showed that lymphocytes which have not been sensitized to the tissue antigen accumulate in the inflammatory reaction. Further evidence for this was obtained by showing that ^{3}H-uridine–labeled lymph node cells from pertussis-sensitized donors migrated to the adrenal heat lesion when transferred together with unlabeled cells from adrenal-sensitized donors, and to the brain heat lesion when transferred together with unlabeled cells from spinal cord-sensitized donors.

Although the results discussed so far clearly show that lymphocytes stimulated by irrelevant antigens accumulate in the autoimmune lesions, they do not exclude the possibility that there is preferential accumulation of specifically sensitized cells in the appropriate lesion. Accordingly, experiments were designed to test this possibility. Recipients were given lymph node cells which had been labeled in vitro with ^{3}H-thymidine or ^{3}H-adenosine from donors sensitized to one tissue antigen and an equal number of unlabeled cells from donors sensitized to the other tissue antigen. Labeled cells were found in significant numbers at 24 and 48 hr in the infiltrates around the heat lesions. Moreover, they were found in equal numbers around each lesion, regardless of which type of donor cells had been labeled. The results thus failed to provide evidence for preferential accumulation of labeled donor lymphocytes in the appropriate site. Nevertheless, the question may still be asked: do these results definitely eliminate the possibility that there is a preferential accumulation of specifically sensitized cells around the specific antigen? Two considerations are particularly relevant to this question. The first is that the methods used for tagging the donor cells obviously will not selectively label the cells sensitized to the tissue antigen, but will also label cells with specificities towards antigens of the two strains of *Mycobacteria* and *Bordetella pertussis* and undoubtedly cells with many other specificities as well. Thus it seems likely that the proportion of cells with the appropriate tissue specificity among those labeled is quite

small. The second consideration is that when the infiltrate around the recipient's heat lesion starts developing, it leads to the accumulation of lymphocytes of unrelated specificities, as already discussed. Thus, it must be concluded that a preferential accumulation of labeled specifically sensitized cells might occur in this experiment but be concealed by the simultaneous accumulation of labeled donor cells with other specificities. An attempt was therefore made to block the vast influx of cells into the lesions by irradiation of the recipients with 400 R 1 day before transfer of the two types of donor cells, those of one type labeled; very little inflammation developed in the lesions of these recipients. Labeled cells were present in high, but equal numbers in the adrenal and brain lesions. It must therefore be concluded that if a preferential accumulation of specifically sensitized cells occurs at all, it is not detectable with the methods used and therefore probably of a low order numerically. Despite these considerations the most straightforward explanation of the findings is that there is no selective accumulation and that sensitized cells with specificity towards each tissue antigen are present to the same extent in the two sites. Furthermore, it seems likely that they are present in the infiltrate in the same proportion as they are represented in the circulation among the type of newly formed lymphocyte which is prone to enter such lesions.

Despite the failure to demonstrate preferential accumulation of labeled donor cells in the appropriate lesion, it is presumed that the reaction is initiated by contact between specifically sensitized cells and the tissue antigen. The mechanism responsible for this contact is unknown. This step could be entirely nonspecific immunologically; thus, a few lymphocytes might emigrate into an area of slight inflammation bordering the heat lesion or possibly, as postulated by Koster and McGregor, (15) there may be a continuous extravascular migration of lymphocytes.

As indicated in the introduction, studies on the specificity of the cellular infiltrate in delayed-sensitivity reactions have yielded results similar to those reported here. However, in comparison with the autoimmune lesions, delayed reactions generally show a considerably higher percentage of monocytes or macrophages (17, 18). This fact, as well as certain in vitro observations of factors affecting macrophages in delayed hypersensitivity (migration-inhibitory factor and chemotactic factor), have focused attention on the secondary accumulation of monocytes and macrophages and the role of the lymphocytes has been largely ignored. However, review of the experiments referred to indicates that there is no convincing evidence of preferential accumulation of specifically sensitized cells even among the lymphocytes in delayed reactions, although it must be pointed out that this question has not been critically studied in a system which would permit clear-cut distinction between monocytes and lymphocytes. Studies concerning the specificity of the infiltrate in allografts indicates that here also lymphocytes with unrelated specificities accumulate (19, 20).

What is the role of the recipient's own macrophages in the inflammatory infiltrate? It was shown by Volkman and Gowans (21) and by Spector et al. (22) that macrophages in inflammatory infiltrates stem from blood monocytes produced in the bone marrow, and others have shown that delayed-hypersensitivity reactions develop poorly or not at all in the absence of bone marrow-derived cells (23, 24). The importance of these cells in the present system was clearly demonstrated in experiments where recipients were X-irradiated with 800 R on the day of production of the heat lesions 5 days before transfer of cells from adrenal or spinal cord-sensitized donors. These recipients failed to develop inflammation around the heat lesion in the target tissue. However, recipients which had been restored with bone marrow cells from normal rats or whose tibial bone marrow had been shielded during the irradiation did develop inflammation. This outcome clearly showed that the availability of cells derived from the bone marrow of the recipient is crucial for the development of the allergic inflammation and makes it equally clear that inflammation can develop in the absence of cells from the recipient's lymph nodes and thymus. It should be emphasized that in animals without cells of bone marrow origin not only do monocytes and macrophages fail to accumulate (this is self-evident), but lymphocytes also do not appear around the heat lesion, even though such cells are injected in sufficient numbers to ensure their accumulation in reactions in nonirradiated recipients. In a study by Levine et al. (25) on cellular transfer of allergic encephalomyelitis in Lewis rats it was shown that irradiation of prospective recipients with 400 R partly inhibited transfer and irradiation with 750 R completely inhibited transfer; these findings were confirmed by the present experiments. However, these authors failed in their attempt to restore the ability of the irradiated recipients to develop the allergic inflammation by injection of bone marrow cells in doses far exceeding those used in the present experiments. It is likely that the negative outcome was due to the fact that they injected the bone marrow cells only one day before transfer of donor lymph node cells, whereas we have injected bone marrow cells 5 days before transfer. It has been shown by others (23) that delayed hypersensitivity to tuberculin can be restored to irradiated rats with bone marrow cells, which however, must be in residence for several days.

For the discussion of the mechanisms responsible for the accumulation of cells in the infiltrate it is relevant to recapitulate some recent in vitro studies which have provided information concerning mechanisms responsible for monocyte and macrophage accumulation in delayed hypersensitivity. Thus, it has been shown that when lymphocytes from sensitized animals are incubated with antigen they release a variety of substances, including two which have been shown to affect monocytes or macrophages. One is chemotactic for mononuclear cells (26) and the other, migration-inhibitory factor, inhibits their migration (27, 28). That such preparations have an effect in vivo is indicated by the observation that their intradermal injection is followed by local accumulation of

mononuclear cells[2] (29, 30). It is reasonable to assume that the mechanisms presumed to account for monocyte accumulation in delayed reactions also function in autoimmune lesions. Indeed, David and Paterson (31) have shown that migration-inhibitory factor is released in the supernatant when lymph node cells from rats with allergic encephalomyelitis are incubated with brain antigen. It remains to be shown that this supernatant also contains the factor which is chemotactic for mononuclear cells, but it is reasonable to assume that it does. There is no comparable information from in vitro studies concerning mechanisms affecting lymphocytes and one can only speculate about factors responsible for the influx of these cells into the lesion. It must ultimately depend in some way upon interaction between the tissue-specific antigen and specifically sensitized cells, since only transfer of cells from appropriate donors can initiate the reaction. It might therefore be expected that the continued presence of specifically sensitized cells in the lesion would be essential for the growth of the infiltrate. This, however, is very unlikely in view of the finding that lymph node cells from *pertussis*-sensitized donors were able to accumulate around heat lesions in the brain from which lymphocytes had been almost completely eliminated by X-irradiation 1 day after transfer of cells from spinal cord-sensitized donors, leaving the radioresistant macrophages (32, 33). It is therefore worth considering the possibility that the site of the allergic inflammation is changed for some time as a consequence of having been affected by mediators released from sensitized lymphocytes after contact with antigen in a way that compels newly formed lymphocytes to accumulate. It is conceivable that the change could involve endothelial cells in such a way as to make lymphocytes adhere to them and migrate through them; this has been shown to be the route of emigration of lymphocytes in allergic neuritis in the rat (34). A satisfactory explanation for the accumulation of lymphocytes would have to account for the facts that this can occur in the absence of specifically sensitized lymphocytes in the site, but apparently not in the absence of monocytes or macrophages. If, indeed, the accumulation of lymphocytes in the site is mediated by cells, the macrophages must be responsible. How this might work is unknown.

SUMMARY

The nature and specificity of the mononuclear cells in passively transferred autoimmune encephalomyelitis and adrenalitis were studied. The recipients were prepared by production of a small heat lesion in the target tissue 5 days before transfer. Within 24 hr after transfer of lymph node cells from donors

[2] Hawson, W. T., and D. C. Dumonde. Mediators of cellular hypersensitivity, I. Phlogistic activity of soluble factors generated by lymphoid cell cultures. Presented at British Society for Immunology meeting, November, 1967.

sensitized with the corresponding tissue antigen, a dense mononuclear cell infiltrate developed around the lesion.

When lymph node cells labeled in vitro with ^{3}H-thymidine or ^{3}H-adenosine were transferred, a significant number of labeled lymphocytes was found in the infiltrate at 24 or 48 hr. Lymphocytes labeled with ^{3}H-thymidine showed a greater tendency to accumulate than cells labeled with ^{3}H-adenosine, indicating that newly formed lymphocytes were more prone to enter the reaction than older cells.

Labeled lymphocytes and macrophages of recipient origin and labeled lymphocytes from donors stimulated with *B. pertussis* were also shown to accumulate around the heat lesion provided the reaction had been initiated by transfer of unlabeled lymphocytes from donors sensitized to the appropriate tissue-specific antigen.

In recipients which were given lymph node cells from two groups of donors, sensitized either to spinal cord or to adrenal antigens, with cells from only one group of donors labeled, equal percentages of labeled cells were found around each lesion. Thus, no evidence of preferential accumulation of specifically sensitized lymphocytes was obtained.

In recipients which received whole body irradiation on the day of production of the heat lesions, 5 days before transfer of lymph node cells from appropriately sensitized donors, neither monocytes nor lymphocytes accumulated around the lesion. However, if the tibial bone marrow was shielded or if bone marrow cells were given to the recipients shortly after irradiation, inflammation developed as in normal recipients.

In recipients which were irradiated 24 hr after the transfer of unlabeled lymph node cells from donors sensitized to the appropriate tissue antigen and then given labeled lymph node cells from *B. pertussis*-stimulated donors, labeled lymphocytes were found in the reaction 24 hr later. This accumulation occurred although virtually all the lymphocytes present in the lesion at 24 hr after the first transfer were destroyed by the irradiation.

The results are interpreted as follows. The autoimmune reaction is initiated by the arrival at the site of a few specifically sensitized lymphocytes, probably on a random basis. After contact with antigen, factors are produced and released which cause the influx of monocytes and of lymphocytes, in particular newly formed ones, of various specificities. There is no preferential accumulation of specifically sensitized cells. The influx of lymphocytes appears to require the presence of monocytes or macrophages in the reaction.

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