CONTACT SENSITIVITY IN THE MOUSE

IV. The Role of Lymphocytes and Macrophages in Passive Transfer and the Mechanism of Their Interaction

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Contact sensitivity to 2-phenyl-4-ethoxymethylene oxazolone (oxazolone) is readily produced in mice and can be transferred to normal recipients by peritoneal exudate, lymph node, and bone marrow cells. There is some evidence that this is a mixed immediate and delayed type hypersensitivity reaction with both humoral and cellular components (1, 2). We wished to investigate the ability of purified peritoneal exudate lymphocytes and macrophages to transfer these reactions. Nonspecific swelling (toxic reaction) caused by the application of the contact sensitizing agent to normal mice is a troublesome factor in assessing skin reactions. This difficulty was overcome by using irradiated recipients. It was found that either purified peritoneal exudate lymphocytes or purified macrophages were able to transfer skin reactions to irradiated recipients and that normal macrophages, passively sensitized by lymphocytes within a Millipore chamber (Millipore filter Corp., Bedford, Mass.) were also active.

Materials and Methods

Animals.—CBA mice, 2–3 months old, were obtained from Animal Service Laboratories. Mice of one sex were used in any one experiment. The mice were assigned to experimental and control groups with a table of random numbers.

Immunization (Sensitization) of Donors.—0.1 ml of 3% oxazolone (2-phenyl-4-ethoxymethylene oxazolone, British Drug Houses, Ltd. Poole, Dorset, England) in ethyl alcohol was applied to the skin of the clipped abdomen. In one experiment mice were sensitized with 0.1 ml 7% picryl chloride in ethyl alcohol.

Challenge and Quantification. (1).—The thickness of both ears was measured with an engineer's micrometer (Moore and Wright, Sheffield, 961 M) before and at different times after application of 2 or 3% oxazolone in olive oil. Nonspecific swelling is less in irradiated than in normal recipients and for this reason 3% oxazolone in olive oil was used in most experiments.

Irradiation of Recipients.—The mice were irradiated using the Maximar Unit (Maximar

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Therapeutic Medical X-ray Equipment, Milwaukee, Wis.) at 220 kvp, 15 ma, FSD 60 cm with full back scatter. The filtration was 0.5 mm of Cu and 1.0 mm of Al.

Passive Transfer.—The donors were used 7 days after sensitization. 4 days before transfer 3 ml of Brewer's thioglycollate medium was injected intraperitoneally. Peritoneal exudate cells were obtained by injecting 4 ml of balanced salt solution (BSS) containing 10 units/ml heparin (3), and washing out with 2 ml of BSS. The cells were washed once in BSS and filtered through two layers of No. 110 N Nybolt cloth. The separation of peritoneal lymphocytes and macrophages is described below. Cells were injected intravenously in a volume of 1.0 ml of 1% mouse serum in BSS. In some experiments macrophages and peritoneal exudate cells caused death and occasionally two separate injections were made within 30 min. In later experiments the cells were injected in 10% fetal calf serum (Flow Laboratories, Inglewood, Calif.) inactivated at 56°C for 30 min, and up to 1.5×10^7 macrophages could be injected intravenously without causing death. The mice were challenged 2 hr after injection. Cell viability was measured by dye exclusion using 0.125% eosin (final concentration). All cell populations were at least 95% viable.

Separation of Peritoneal Lymphocytes and Macrophages

Albumin Density Gradient.—The method of Raidt et al. (3) was followed with mino modifications. Bovine serum albumin (Armour fraction V), (Armour and Company, Ltd. Eastbourne, England) was dissolved in BSS. 2 ml of 40% (weight/initial volume) solution was placed in a 20 ml MSE centrifuge tube (Measuring & Scientific Equipment, Ltd., London, England). The washed and filtered peritoneal exudate cells were resuspended in 3 ml of 33% bovine serum albumin and layered above the 40% solution. 3 ml aliquots of 29, 26, 23, 10, and 5% albumin were layered above and the tubes centrifuged at 20,000 *g* (max) for 70 min in the swing out head of the MSE ultracentrifuge. After centrifugation the cells were found at the six interfaces and in the pellet and were recovered with a fine Pasteur pipette. The cells found at the top interface (i.e. the lightest cells) were called fraction A and the cells recovered from lower interfaces and the deposit, fractions B to G. The fractions were diluted in 3–4 volumes of BSS, spun down, and washed once.

Ficoll Density Gradient.—Washed and filtered cells were resuspended in 4 ml of 25% (weight/initial volume) Ficoll (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) in BSS and overlaid with 6 ml of 21%, 5 ml of 16%, and 4 ml of 12% Ficoll. The fractions obtained after centrifugation were called A, B, C, and D; A being the lightest and D the pellet. A hundred million cells were usually placed in each tube and 5×10^7 macrophages found in fractions A and B.[1]

Column Purification of Lymphocytes.—The lymphocytes in fractions C and D were purified with a column. Parker 199 (with penicillin and streptomycin and 0.88 g/liter of $NaHCO_3$: Burroughs Wellcome) gassed with 5% CO_2:air, to bring it to about pH 7, and containing 40% fetal calf serum, was warmed to 37°C and used at that temperature for all the column procedures. The column was made in a 3 cm diameter 50 ml syringe. Rayon wool (Rand-Xerox) was placed in the bottom 2 cm and covered with 7 cm of polystyrene beads (Pontyclan Chemical Co., London, England) in medium and finally with 2 cm of rayon wool. The syringe was then placed in an incubator. The cells were diluted in BSS and spun down and $1–3 \times 10^8$ of them were resuspended in 10–15 ml of medium and warmed to 37°C.

Immediately before use the column was rinsed with 30 ml of medium and the cells then applied drop by drop over a few min. After 20–30 min at 37°C the nonadherent cells (lymphocytes) were eluted with up to 80 ml of medium added drop by drop over about 20

[1] Zembala, M., and G. L. Asherson. The rapid purification of peritoneal exudate macrophages by Ficoll (polysucrose) density gradient centrifugation. Submitted for publication.

min. The cells were spun down and washed once in BSS without added serum. 85–95% of the cells recovered were small lymphocytes. Similar but slightly inferior purification was obtained with peritoneal exudate cells which had not been separated on a density gradient.

Cytology.—Cell preparations were made with the Shandon Cytocentrifuge (Shandon Scientific Co., London, England) (10 min, 300 rpm) and stained with May-Grunwald-Giemsa. Cells were classified as macrophages, lymphocytes, polymorphs, and mast cells. The differential counts are given as a percentage of nucleated cells. Red cells, polymorphs, and mast cells are only found in the pellet fractions.

Millipore Chambers.—Millipore filters (0.1 mμ) were attached to Perspex plastic rings (G. H. Bloore, London, England) (internal diameter 1.2 mm; depth 4 mm) with Tensol cement No. 6 (Imperial Chemical Industries, Teesside, England). Dry heat (80°C, 40 min) on 3 successive days was used for sterilization. Up to 0.3 ml of cells in Parker 199 containing 5% normal mouse serum was placed in each chamber through a hole drilled in the plastic. The hole was closed with a mixture of hard paraffin and vaseline.

Peritoneal exudates were induced in the recipient mice 2 days before the insertion of the Millipore chambers. The chambers were placed in the right lower quadrant of the abdomen through a midline incision under nembutal anesthesia. The abdomen was closed using Michelle clips. The mortality was less than 5%.

Bacteriological Test for Defective Chambers.—The integrity of the chambers was tested by incubation after use in Robertson's cooked meat medium inoculated with *Clostridium sporogenes*. After shaking for 36 hr the chambers were removed and the fluid within examined by Gram stain. This may be an adequate test of the integrity of the chambers as *Cl. sporogenes* is a motile organism which produces proteolytic enzymes.

Statistics.—The Mann-Whitney U test was used. Statistically significant refers to $P \leq 0.05$ in a double tail test.

RESULTS

Effect of Irradiation on Skin Reactions

Effect of Irradiation on Nonspecific Swelling (Table I).—The swelling caused by painting the ears of unsensitized mice with oxazolone is called nonspecific. The following experiment illustrates the depression of nonspecific swelling by irradiation.

Groups of five mice were irradiated with 500, 850, or 1000 R or left unirradiated. 7 days later they were challenged with 2% oxazolone and the increase in ear thickness was measured 24 hr later in units of 10^{-3} cm. Table I shows that the unirradiated mice gave 1.8 units of nonspecific swelling while those irradiated with 850 and 1000 R gave -0.6 and 0.8 units of nonspecific swelling. It was concluded that irradiation reduced nonspecific swelling.

Facilitation of Passive Transfer by Irradiation of Recipients (Table II).—Fewer lymph node cells were required to transfer a secondary response to irradiated recipients than to normal recipients (4). This suggested that irradiation of the recipient might also facilitate the passive transfer of skin reactions by peritoneal exudate cells. Peritoneal exudate cells from donor mice sensitized to oxazolone were transferred to normal and irradiated recipients. The ears of the recipients were then challenged with oxazolone and the increment of ear thickness was measured 24 hr later. Table II shows that as few as 1×10^6

peritoneal exudate cells transferred significant reactions to irradiated recipients. In contrast 3×10^6 peritoneal exudate cells failed to transfer significant reactions to unirradiated recipients although 10×10^6 cells were effective.

This experiment suggested that irradiation facilitated the passive transfer of skin reactions by peritoneal exudate cells. The alternative interpretation that peritoneal cells restored the defect in inflammatory responses in the irradiated

TABLE I

Effect of Irradiation on Nonspecific Swelling

Irradiation	Increment of ear thickness at 24 hr
R	
0 (control)	1.8 ± 0.91
500	1.3 ± 0.24
850	−0.6 ± 0.84
1000	0.8 ± 1.2

Groups of five mice were irradiated 7 days before challenge with 2% oxazolone. The figures show the increment of ear thickness ± the standard deviation in units of 10^{-3} cm.

TABLE II

The Effect of Irradiation of the Recipient on Passive Transfer

Cells transferred		Irradiated recipients	Normal recipients
1×10^7 PE cells	(Ox sens)	5.3 (1)	3.8 ± 0.28 (5)
3×10^6	(Ox sens)	2.7 ± 0.69 (3)	1.8 ± 0.64 (3)
1×10^6	(Ox sens)	3.3 ± 1.67 (3)	1.9 ± 0.63 (3)
1×10^7 PE cells	(Pic sens) -ve control	0.2 ± 0.075 (3)	
3×10^6	(Pic sens) -ve control	0.1 ± 0.12 (3)	
1×10^6	(Pic sens) -ve control	0.1 ± 0.069 (3)	
Nil	-ve control	0.15 ± 0.16 (5)	1.7 ± 1.09 (3)

The figures show the increment of ear swelling at 24 hr in units of 10^{-3} cm. The number of mice on which they are based is shown in parentheses. The recipient mice were irradiated the day before the passive transfer (intravenously) of peritoneal exudate (PE) cells from donors sensitized to oxazolone (Ox sens). Cells from mice sensitized to picryl chloride (Pic sens) served as negative controls. The mice were challenged with 2% oxazolone.

recipients (and hence restored nonspecific swelling) was excluded by control experiments.

In the first experiment irradiated recipients were given peritoneal exudate cells from mice sensitized to picryl chloride. Table II shows that 1×10^7 peritoneal exudate cells from donors sensitized to picryl chloride failed to restore nonspecific swelling to oxazolone. Other control experiments using cells from unsensitized donors gave similar negative results and are shown in Table III. Because of these results irradiated recipients were used in all subsequent experiments. The standard dose was 850 R.

Passive Transfer of Skin Reactions into Irradiated Mice by Peritoneal Exudate Macrophages and Lymphocytes

Cell Populations Used for Passive Transfer.—Purified peritoneal macrophages (*purified macrophages*) which were better than 98% pure were obtained from the

TABLE III

Passive Transfer by Peritoneal Exudate Cells after Albumin Density Gradient Centrifugation

Experiment	Cells	No. of cells	Increment of ear thickness at		
			12 hr	24 hr	48 hr
1. Sensitized donors*	PE cells	1.5×10^7		8.8 ± 0.87	7.6 ± 0.38
	PE cells pooled after centrifugation	1.5×10^7		8.4 ± 0.43	6.4 ± 0.66
	Nil (-ve control)			1.2 ± 0.65	1.7 ± 0.41
2. Sensitized donors‡	Fr. A+B (purified macrophages) (M > 99%)	3×10^6	5.2 ± 0.79	4.8 ± 0.50	3.8 ± 0.58
	Fr. C (purified macrophages) (M > 99%)	4×10^6	5.5 ± 0.36	5.4 ± 0.38	4.1 ± 0.94
	Fr. D (purified macrophages) (M 99%)	5×10^6	7.2	5.9	4.3
	Fr. E (M 97%; L 2.6%)	3×10^6	5.2 ± 1.04	5.4 ± 0.46	4.1 ± 1.11
	Fr. F (lymphocyte-rich fraction) (M 90%; L 10%)	6×10^6	3.4 ± 0.42	6.6 ± 0.23	4.7 ± 0.82
	Fr. G (lymphocyte-rich fraction) (M 25%; L 50%)	3×10^6	3.6 ± 0.48	7.1 ± 0.57	5.8 ± 0.24
	Nil (-ve control)		0.6 ± 0.73	1.5 ± 0.24	1.5 ± 0.58
	PE cells (M 80%; L 12%)	1×10^6	2.5 ± 0.92	4.4 ± 0.49	3.6 ± 0.78
	PE cells	3×10^6	3.4 ± 1.53	5.2 ± 0.98	3.5 ± 1.88
	PE cells	6×10^6	4.6 ± 0.44	6.6 ± 0.53	6.1 ± 0.78
3. Unsensitized donors§	Fr. A+B (purified macrophages) (M 99%)	5×10^6	1.0 ± 1.9	1.9 ± 0.72	
	Fr. C (purified macrophages) (M 99%)	7×10^6	0.3 ± 0.50	0.9 ± 0.59	
	Fr. D (purified macrophages (M 98%; L 1%)	3×10^6	0.3 ± 0.38	0.6 ± 0.77	
	Fr. E (M 94%; L 6%)	7×10^6	0.6 ± 0.16	1.6 ± 1.36	
	Fr. F (lymphocyte-rich fractions) (M 65%; L 28%)	3×10^6	1.1 ± 0.65	1.8 ± 0.78	
	Fr. G (lymphocyte-rich fractions) (M 23%; L 23%)	3×10^6	0.5 ± 0.40	1.4 ± 0.59	
	PE cells	3×10^6	1.0 ± 0.91	1.6 ± 0.71	
	Nil (-ve control)		0.7 ± 0.15	1.6 ± 0.27	

Each figure (in units of 10^{-3} cm) is based on three mice except the second and third figure of Experiment 1 (four mice) and the third figure of Experiment 2 (one mouse).

* Peritoneal exudate (PE) cells from sensitized donors were injected either after storage at 4°C or after albumin density gradient centrifuge separation followed by recombining the fractions. In this and subsequent experiments 3% oxazolone was used for challenge.

‡ In this and subsequent experiments the differential count on the fractions (Fr.) is shown. M and L stand for macrophages and lymphocytes.

§ Peritoneal exudate cells were taken from unsensitized donors as a control for the effect of unsensitized cells on nonspecific swelling.

fractions A–D of the albumin density gradient and fractions A and B of the Ficoll density gradient. The lymphocyte-rich fractions refer to fractions F and G (pellet) of the albumin density gradient, and fraction D (pellet) of the Ficoll density gradient. They contained 10–30% and sometimes more small lymphocytes.

Purified peritoneal lymphocytes (*purified lymphocytes*) which were 85–95% pure were obtained by passing the lymphocyte-rich fractions (or occasionally the initial exudate) through a column of rayon wool and polystyrene beads. Because lymphocytes and macrophages show different behavior in passive transfer experiments the differential counts of the fractions used are given in the tables.

Passive Transfer by Peritoneal Exudate Cells after Albumin Density Gradient Centrifugation (Table III).—The first experiment showed that 1.5×10^7 peritoneal exudate cells separated on an albumin density gradient and then recombined were as active, in passive transfer, as the same number of the original peritoneal exudate cells kept in the cold. The second experiment showed

TABLE IV

Time Course of Ear Swelling: Difference between the Ear Thickness at 12 and 24 Hr Following Passive Transfer of Purified Peritoneal Macrophages and Lymphocyte-Rich Fractions

Fraction	Experiment				Mean
	1	2	3	4	
A–D (purified macrophages)	−0.3*	−0.2	−0.3*	−0.9	−0.4
E	+0.2	−0.1	—	—	+0.1
F (lymphocyte-rich fractions)	+3.2	+1.1	+0.9	+2.3‡	+1.9
G (lymphocyte-rich fractions)	+3.5	+0.7	+2.2	—	+2.1
Nil (-ve control)	+0.9	+0.2	+0.7	+0.3	+0.5

Cell fractions ($1–5 \times 10^6$) from sensitized donors were transferred to groups of three or more irradiated recipients. The figures show the difference between the thickness of the ears (in units of 10^{-3} cm) at 12 and 24 hr in four separate experiments. Experiment 1 is taken from Table III. A positive difference means that the ear swelled between 12 and 24 hr and a negative difference means that the ear shrank.

* A + B.

‡ F + G.

that both purified macrophages and lymphocyte-rich fractions from an albumin density gradient transferred ear swelling. The third (control) experiment showed that separated cells from unsensitized donors did not affect ear swelling.

Time Course of Ear Swelling Following Passive Transfer of Purified Peritoneal Lymphocytes and Macrophages (Tables III, IV, and V).—Table III, Exp. 2, shows that ear swelling caused by passive transfer of peritoneal exudate cells increases linearly when 1, 3, or 6×10^6 cells are given and that the increase in the swelling between 12 and 24 hr (1.8–2.1 units) was independent of the number of cells given. It is therefore interesting that macrophages purified by albumin density gradient centrifugation transferred reactions which were somewhat greater at 12 hr than at 24 hr after challenge, while the lymphocyte-rich fractions (F and G) transferred reactions which were greater at 24 hr than at 12 hr. These results were confirmed in three additional experiments which are

summarized in Table IV. In subsequent experiments peritoneal exudate cells were separated on a Ficoll density gradient and the lymphocyte-rich fraction was usually purified on a column. Table V shows that purified macrophages (fractions A and B) which were 99% pure transferred ear swelling which was greater at 12 hr than at 24 hr, while purified lymphocytes (83% pure) transferred reactions which were greater at 24 hr than at 12 hr. This difference in time course suggested that there might be a corresponding difference in the mechanism of passive transfer by macrophages and lymphocytes.

Interaction between Lymphocytes and Macrophages

Millipore Chamber Experiment in Normal Recipients (Table VI).—The previous section showed that both purified peritoneal lymphocytes and macrophages

TABLE V

Passive Transfer by Purified Peritoneal Macrophages and Purified Peritoneal Lymphocytes (Ficoll Gradient Separation)

Cells	No. of cells	Increment of ear thickness	
		12 hr	24 hr
Fractions A + B (purified macrophages) (M 99%; L 1%)	6×10^6 (5)	3.2 ± 0.67	2.5 ± 0.93
Purified lymphocytes (M 7%; L 83%)	5×10^6 (4)	3.4 ± 0.88	4.3 ± 0.58
Nil control	(4)	0.8 ± 0.29	0.4 ± 0.43

M and L, macrophages and lymphocytes respectively. The number of mice is shown in parentheses. The lymphocytes were concentrated in fractions C and D of the gradient and purified on a column. The 12 and 24 hr readings (in units of 10^{-3} cm) were made on different mice.

transferred skin reactions. It is commonly held that lymphocytes are immunologically competent while macrophages are immunologically incompetent. This raised the problem of how peritoneal macrophages from sensitized donors transfer skin reactions. It seemed possible that lymphocytes from sensitized donors produced a factor (e.g. cytophilic antibody) which enabled normal macrophages to transfer skin reactions.

In the first experiment various cells from donors sensitized to oxazolone were placed in Millipore chambers. The chambers were then inserted into normal unirradiated mice bearing a thioglycollate exudate. Control mice received chambers which contained normal cells.

Table VI shows that mice bearing Millipore chambers which contained lymph node lymphocytes or purified peritoneal lymphocytes from sensitized donors gave statistically significant ear swelling when challenged with oxazolone.

The controls were negative and mice bearing chambers containing lymph node lymphocytes or peritoneal exudate cells from unsensitized donors did not

show ear swelling. Purified macrophages from donors sensitized to oxazolone were also inactive when placed within the Millipore chamber. This preliminary experiment suggested that lymph node lymphocytes and purified peritoneal lymphocytes produced a factor which was able to pass through a Millipore filter and convey skin reactions to oxazolone. It also suggested that purified macrophages were unable to produce this factor.

Millipore Chamber Experiments in Intermediate Recipients (Tables VII and VIII).—Further experiments were designed: (*a*) to confirm that lymphocytes from donors sensitized to oxazolone produced a factor which was able to pass

TABLE VI

Millipore Chamber Experiment: Ear Swelling in Unirradiated Mice Bearing Millipore Chamber

Cell in Millipore chamber	No. of cells	Increment of ear thickness in mice bearing the Millipore chamber	
		12 hr	24 hr
Lymph node lymphocytes (ox sens)	2.4×10^7	1.5 ± 0.83 (4)	2.2 ± 0.98 (4)
Purified PE lymphocytes (ox sens) (M 0.5%; L 99%)	7×10^6	2.4 ± 0.82 (5)	3.5 ± 1.20 (4)
Purified macrophages (ox sens) (M 99%; L 1%)	6×10^6	0.9 ± 0.67 (4)	0.9 ± 0.46 (2)
Lymph node lymphocytes (normal)	2.3×10^7	0.6 ± 0.18 (2)	0.7 ± 0.04 (2)
PE cells (normal)	8.4×10^6	0.7 (1)	1.2 (1)

Cells from normal mice and mice sensitized to oxazolone (Ox sens) were placed in Millipore chambers. These were inserted into normal mice bearing a 2 day peritoneal exudate (PE). The mice were challenged 44 hr after operation. The figures show the increment of ear thickness (in units of 10^{-3} cm) at 12 and 24 hr in different groups of mice. The number of mice is shown in parentheses. M and L, macrophages and lymphocytes respectively.

through a Millipore filter and convey skin reactions to oxazolone; (*b*) to confirm that macrophages from donors sensitized to oxazolone failed to produce a similar factor; (*c*) to investigate which cells (macrophages or lymphocytes) were able to acquire the ability to transfer skin reactions from sensitized lymphocytes within a chamber; (*d*) to investigate whether the serum of mice with Millipore chambers acquired the ability to transfer skin reactions.

Purified peritoneal lymphocytes from donors sensitized to oxazolone were placed in Millipore chambers. These were inserted into the peritoneal cavity of intermediate recipients bearing a thioglycollate exudate. The exudate was harvested from some mice 18 hr and from others 40 hr later and macrophages and lymphocytes separated on a Ficoll density gradient. The ability of these cells to transfer ear reactions was tested by injecting them into irradiated final

recipients. Table VII shows that peritoneal lymphocytes inside the chamber transferred reactivity to macrophages outside the chamber within 18 hr. It also shows that only the macrophages of the intermediate recipient transferred reactions while the lymphocyte-rich fraction and the serum of the intermediate recipients were inactive. Similar data, which are not shown in the table, were obtained when 1.8×10^7 lymph node lymphocytes were placed in the chamber.

These results were confirmed in Table VIII. Purified peritoneal lymphocytes, lymph node lymphocytes, or purified macrophages from donors sensitized to oxazolone, were placed in Millipore chambers. These were inserted into inter-

TABLE VII

Millipore Chamber Experiments Using Intermediate and Final Recipients: Ear Swelling in Irradiated Final Recipients Receiving Cells from Intermediate Recipients Bearing Millipore Chamber

5×10^6 purified peritoneal lymphocytes (L 96%; M 4%) from donors sensitized to oxazolone were placed in Millipore chambers in intermediate recipients.

Cells transferred from intermediate recipients to final recipient	No. of cells $\times 10^6$ Transfer 18 hr	40 hr	Cells taken 18 hr after insertion of chamber Increment of ear thickness in final recipient 12 hr	24 hr	Cells taken 40 hr after insertion of chamber Increment of ear thickness in final recipient 12 hr	24 hr
Fr. A+B (purified macrophages) (M > 98%; L 1%)	3	3	2.2 (1)	2.9	1.5 ± 0.66 (3)	2.3 ± 0.80
Fr. C (M 99-89%; L 1-8%)	1.8	5	1.6 ± 0.6 (2)	2.4 ± 0.14	1.6 (1)	2.3
Fr. D (purified lymphocytes) (M 10-7%; L 80-36%)	8	18	0.5 ± 0.27 (3)	1.2 ± 0.21	0.3 ± 0.67 (3)	0.6 ± 0.26
Serum 0.5 ml			0.5 (1)	1.0	0.2 ± 0.08 (3)	0.6 ± 0.26
No cells (-ve control)			0.45 ± 0.27 (3)	1.3 ± 0.33	0.15 ± 0.18 (5)	0.7 ± 0.34

Cells (purified peritoneal lymphocytes) from donors sensitized to oxazolone were placed in Millipore chambers which were inserted into intermediate recipients bearing 2-day exudates. The exudates were taken either 18 or 40 hr after the insertion of the chamber and injected into irradiated final recipients. The table shows the increment of ear thickness (in units of 10^{-3} cm) in the final recipients 12 and 24 hr after challenge. A synopsis of this table appears in reference 5. A parallel experiment with lymph node lymphocytes (1.8×10^7) within Millipore chambers gave similar results.

Fr. stands for fraction. M and L, macrophages and lymphocytes respectively.

mediate recipients bearing a thioglycollate exudate. The exudate was harvested 42 hr later and cells from the peritoneal exudate of the intermediate recipients were transferred to the final recipient. Table VIII shows that peritoneal and lymph node lymphocytes within the chamber conveyed reactivity to peritoneal exudate cells outside the chamber. In contrast, peritoneal macrophages (from sensitized donors) within the chamber were entirely inactive. Table VIII also shows that purified peritoneal macrophages outside the chamber transferred reactions while twice their number of purified peritoneal lymphocytes were inactive. The serum from the intermediate recipients was also inactive.

It was provisionally concluded that: (*a*) lymphocytes from donors sensitized to oxazolone produced a factor (presumptive cytophilic antibody) able to pass

TABLE VIII

Millipore Chamber Experiment Using Intermediate and Final Recipients Ear Swelling in Irradiated Final Recipients Receiving Cells from Intermediate Recipients Bearing Millipore Chamber

Cells transferred from intermediate recipients to final recipients	No. of cells × 10^6 in experiment			Experiment 1 Lymph node lymphocytes in Millipore chamber of intermediate recipient Increment of ear swelling		Experiment 2*a* Purified peritoneal lymphocytes in Millipore chamber of intermediate recipient Increment of ear swelling			Experiment 2*b* Purified peritoneal macrophages in Millipore chamber of intermediate recipient Increment of ear swelling	
	1*a*	2*a*	2*b*	12 hr	24 hr	12 hr	24 hr	48 hr	24 hr	48 hr
Purified macrophages (M > 99%)	4	4.3	8.4	2.3 ± 0.66	3.0 ± 0.59 (5)	1.7 ± 0.50	2.7 ± 0.29	3.2 ± 0.36 (4)	0.8 ± 0.27	1.4 ± 0.27 (5)
Purified macrophages (M > 99%)	6	—	—	2.0 ± 0.50	2.8 ± 0.34 (5)					
Purified peritoneal lymphocytes (L 95%; M 2%)	9	11	9.6	0.15 ± 0.32	1.0 ± 0.32 (4)	0.7 ± 0.27	0.8 ± 0.37	0.85 ± 0.29 (4)	0.7 ± 0.22	1.3 ± 0.52 (4)
PE cells	5	—	—	1.5 ± 0.68	1.7 ± 1.05 (3)					
PE cells	9	9	11	2.5 ± 0.63	3.6 ± 0.35 (3)	2.1 ± 0.91	3.0 ± 0.81	3.2 ± 0.76 (4)	0.8 ± 0.22	1.3 ± 0.30 (5)
Serum				0.8 ± 0.87	1.6 ± 0.57 (4)	0.5 ± 0.23	0.8 ± 0.35	0.9 ± 0.51 (6)	1.1 ± 0.36	1.7 ± 0.35 (4)
Nil (-ve control)				0.4 ± 0.29	1.1 ± 0.55 (4)	0.6 ± 0.34	0.9 ± 0.33	1.1 ± 0.57 (6)	0.8 ± 0.48	1.4 ± 0.58 (3)

In Experiment 1 cells (2.4×10^7 lymph node lymphocytes) from donors sensitized to oxazolone were placed in Millipore chambers which were inserted into intermediate recipients bearing 2-day exudates. The exudates were taken 42 hr after the insertion of the chamber and injected into irradiated final recipients.

In Experiment 2 *a* cells (3.2×10^6 purified peritoneal lymphocytes L 39%; M 3%) from donors sensitized to oxazolone were placed in Millipore chambers. In Experiment 2 *b* 7×10^6 purified macrophages (> 99% M) from the same donors were used. The remainder of this experiment followed the plan of Experiment 1.

The increment of ear swelling was measured in units of 10^{-3} cm; M and, L macrophages and lymphocytes respectively; PE, peritoneal exudate.

through a Millipore filter and convey skin reactions to oxazolone; (*b*) macrophages failed to produce a similar factor; (*c*) peritoneal macrophages outside the chamber were modified by the factor (presumptive cytophilic antibody) and acquired the ability to transfer skin reactions; (*d*) lymphocytes outside the chamber were not modified in a similar way; (*e*) the factor (presumptive cytophilic antibody) did not appear in the serum of the intermediate recipients as judged by serum transfer.

Control Experiments (*Table IX*).—It was tacitly assumed in the last paragraph that macrophages from intermediate recipients bearing chambers con-

TABLE IX

Millipore Chamber Experiment in Intermediate Recipients: Ear Swelling in Irradiated Final Recipients Receiving Cells from Intermediate Recipients Bearing a Millipore Chamber (Control Experiment)

Cells transferred from intermediate recipient to final recipient	No. of cells transferred to final recipient	Experiment 1 *a* Purified peritoneal lymphocytes from donors sensitized to Oxazolone in chamber. (2.3×10^6): L 95%; M 3% Increment of ear thickness in final recipient*		Experiment 1 *b* Purified peritoneal lymphocytes from donors sensitized to picryl chloride in chamber. (3.6×10^6) Increment of ear thickness in final recipient*	
		12 hr	24 hr	12 hr	24 hr
Purified macrophages (M 99%; L 1%)‡	5.5×10^6 (6)	2.4 ± 0.36	2.6 ± 0.59 (5)	0.55 ± 0.18	0.5 ± 0.46
Purified macrophages (M 99%; L 1%)	8 $\times 10^6$ (6)	2.5 ± 0.26	2.7 ± 0.47 (5)	0.5 ± 0.42	0.6 ± 0.21
Purified peritoneal lymphocytes (L 94%; M 1%)	12.5×10^6 (4)	0.35 ± 0.5	0.8 ± 0.7 (4)	0.55 ± 0.26	0.8 ± 0.46
Serum			(4)	0.4 ± 0.17	0.75 ± 0.41
Nil (-ve control)	(5)	0.7 ± 0.34	1.0 ± 0.16	0.7 ± 0.34§	1.0 ± 0.16§

In Experiment 1 *a*, cells (purified peritoneal lymphocytes) from mice sensitized to oxazolone were placed in Millipore chambers. In the control experiment (Experiment 1 *b*) comparable cells from mice sensitized to picryl chloride were placed in Millipore chambers. The rest of the experiment followed the outline in the legend to Table VIII. Note that all the final recipients were challenged with oxazolone and not with picryl chloride.

* The increment of ear thickness is measured in units of 10^{-2} cm.

‡ L and M, lymphocytes and macrophages respectively.

§ The same nil controls (which did not receive any cells) were used in the two parts of the experiment.

taining normal or other control cells were unable to restore nonspecific swelling to irradiated recipients and that leakage of cells from the chambers either did not occur or was unimportant.

The second column of Table IX shows that macrophages from intermediate recipients bearing Millipore chambers containing cells from donors sensitized to picryl chloride failed to restore nonspecific swelling. This suggested that the lymphocyte-macrophage interaction was immunologically specific.

The first column of Table IX shows the relevant positive control and confirms that chambers containing purified peritoneal lymphocytes from donors sensitized to oxazolone conveyed reactivity to macrophages outside the chamber.

The integrity of the chambers in this experiment were tested by incubation with *Clostridium sporogenes* (see Materials and Methods). 3 of the 14 chambers containing lymphocytes from donors sensitized to picryl chloride, and 2 of the 7 chambers containing lymphocytes from donors sensitized to oxazolone, were leaky.

DISCUSSION

Contact sensitivity is a delayed hypersensitivity phenomenon as judged by its histology and its passive transfer by lymph node and peritoneal exudate cells. However, in the mouse, contact sensitivity shows both humoral and cellular components (mixed reaction) as judged by the occurrence of polymorphs in the lesion and swelling as early as 4 hr after challenge.

In the mouse, contact sensitivity to oxazolone can be passively transferred by peritoneal exudate cells. The present experiments show that two cell populations are active: purified peritoneal macrophages and purified peritoneal lymphocytes. Three technical points were important.

Quantitation.—Skin reactions were assessed by measuring ear thickness before and after challenge with an engineer's micrometer. This provided figures which could be handled statistically.

Cell Separation.—Density gradient centrifugation provided macrophages which were 98 to better than 99% pure and had not undergone prolonged incubation at 37°C or treatment with proteolytic enzymes.

Irradiation of Recipients.—Unsensitized mice show nonspecific ear swelling of up to 6 units when challenged with 2% oxazolone. This background may mask small degrees of swelling following passive transfer. This nonspecific swelling was reduced to about 1 unit or less by irradiation at 850 r even when 3% oxazolone was used for challenge.

There was also a suggestion that irradiation actually increased swelling following passive transfer. The possibility that passive transfer might be confused with the restoration of nonspecific swelling to oxazolone by peritoneal exudate cells was excluded by control experiments.

The evidence that skin reactions can be transferred by two different cell populations was based initially on the difference in the morphology of the cell populations and in the time course of the swelling. The finding that both lymphocyte and macrophage populations transfer skin reactions is puzzling as macrophages are not antibody-producing cells (see reference 6) although they possess receptors for cytophilic antibodies. (7) This suggested that lymphocytes might release an antibody which was cytophilic for macrophages.

Millipore chamber experiments supported this hypothesis. However, as 5 out of 21 chambers in one experiment were leaky, certain other interpretations of these experiments should be considered.

(*a*) Lymphocytes escaped from the chambers and were injected into the

final recipients. This was excluded by the finding that lymphocytes outside the chamber were inactive even in large numbers.

(*b*) Lymphocytes escaped from the chambers, acquired the density and morphology of macrophages, and were injected into the final recipients. This is unlikely on quantitative grounds unless nearly all the lymphocytes escaped from the chamber, remained in the peritoneal cavity, and acquired the characteristics of the macrophages.

(*c*) Lymphocytes escaped from the chamber and conveyed factors to contiguous macrophages. This is unlikely as macrophages are able to acquire presumptive cytophilic antibody from serum without coming in contact with lymphocytes,[2] but it cannot be excluded.

In spite of these reservations, the Millipore chamber experiments showed that lymphocytes from sensitized donors conveyed a factor to macrophages which enabled them to transfer skin reactions and that this factor was probably able to pass through a Millipore membrane.

These experiments provide further evidence of the differences in behavior between peritoneal macrophage and peritoneal lymphocyte populations. Some of these differences are: (*a*) Lymphocytes but not macrophages from sensitized donors produce a factor which enables normal peritoneal exudate macrophages to transfer skin reactions. (*b*) Macrophages but not lymphocytes are able to acquire this factor. (*c*) Treatment with trypsin of macrophages from donors sensitized to oxazolone abolishes their ability to transfer skin reactions. Trypsin has only a transient effect on transfer by peritoneal lymphocytes.[2] (*d*) Treatment with anti-mouse gamma globulin serum of macrophages from donors sensitized to oxazolone abolishes their ability to transfer but has no effect on lymphocytes.[2]

These differences between lymphocyte and macrophage populations suggest that they transfer skin reactions by different mechanisms.

Skin reactions transferred by lymphocytes are regarded as examples of delayed-type hypersensitivity (cell-mediated immunity) with the caveat that lymphocytes might transfer immediate type hypersensitivity by producing a circulating antibody. The present observations show that macrophages are also able to transfer skin reactions and may acquire this capacity from lymphocytes. Blazkovec *et al.* (8) reported local passive transfer of skin reactions with a partially purified preparation of macrophages which they attributed to contamination by lymphocytes. Turk and Polak (9) produced reactions with a highly purified population of macrophages which had some of the characteristics of the Arthus reaction. Bennett (10), and Granger and Weiser (11) de-

[2] Zembala, M., and G. L. Asherson. Contact sensitivity in the mouse. V. The role of macrophage cytophilic antibody in passive transfer and the effects of trypsin and anti-gamma globulin serum. To be submitted for publication

scribed in vivo and in vitro inhibition of tumor growth by macrophages from immune donors.

The present experiments show that macrophages also transfer reactions after systemic (intravenous) injection. This suggests that in actively sensitized animals there are three modes of immunological tissue damage: hypersensitivity mediated by lymphocytes (classical delayed hypersensitivity), hypersensitivity mediated by circulating antibody (classical immediate type hypersensitivity), and hypersensitivity mediated by macrophages which have passively acquired cytophilic antibody (macrophage-mediated hypersensitivity).

SUMMARY

Contact sensitivity skin reactions were produced in mice by immunization with 2-phenyl-4-ethoxymethylene oxazolone (oxazolone) and detected by the increase in ear thickness after challenging the ears with 2% oxazolone. These skin reactions can be transferred from immunized donors to irradiated recipients by peritoneal exudate cells induced by thioglycollate.

The peritoneal exudate cells were separated into purified macrophage and purified lymphocyte populations. Both cell populations transferred skin reactions. However, their time course was different. The reactions produced by lymphocytes were greater at 24 hr than at 12 hr while the reactions produced by macrophages declined slightly between 12 and 24 hr.

The working hypothesis was formed that the peritoneal lymphocytes conveyed a factor (presumptive cytophilic antibody) to peritoneal macrophages which enabled them to transfer ear reactions. Experiment showed that peritoneal and lymph node lymphocytes from sensitized donors within a Millipore chamber conveyed a factor to macrophages outside the chamber which enabled them to transfer ear reactions. In contrast, peritoneal macrophages (from sensitized donors) within the chamber and peritoneal lymphocytes outside the chamber were inactive.

These findings suggested that there are three modes of immunological tissue damage: hypersensitivity mediated by lymphocytes (classical delayed hypersensitivity), hypersensitivity mediated by circulating antibody (classical immediate type hypersensitivity), and hypersensitivity mediated by macrophages which have passively acquired a factor (macrophage-mediated hypersensitivity).

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