

IN VITRO GENERATION OF MEMORY LYMPHOCYTES REACTIVE TO TRANSPLANTATION ANTIGENS*

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After rejection of tissue allografts, animals acquire immunological memory to the transplantation antigens of the graft (1). The memory thus acquired manifests itself in several distinct reactions: (a) Accelerated rejection of a second graft antigenically similar to the first transplant (second set effect) (1). (b) Enhanced rejection of tissue grafts by normal animals receiving lymphocytes from immunized donors (1, 2). (c) Greater efficiency in breakdown of tolerance in animals receiving lymphocytes from immunized donors (3). (d) Accelerated transformation of lymphocytes re-exposed to the sensitizing cells in vitro (4).

The immunological memory has been attributed to the clonal progeny of lymphocytes which responded to the antigens of the primary graft. Thus, the increase in number of specific antigen-reactive cells seemed to account for the enhanced response in sensitized animals. On the basis of this, one would expect lymphocytes from animals presensitized to histocompatibility antigens to react more vigorously than normal lymphocytes in both graft vs. host and the mixed lymphocyte culture reactions. However, this is not the case (4, 5), suggesting that immunological memory may be based on changes in the lymphocytes themselves, rather than on an increased number of specific antigen-reactive lymphocytes (6).

To analyze the behavior and properties of memory cells, one needs pure populations of such cells, preferably in an in vitro system. In previous studies (7-9) we showed that blast cells, obtained by stimulation of rat lymphocytes with phytomitogens, redifferentiate, after removal of the mitogen, to small lymphocytes termed "secondary lymphocytes." Studies on the pattern of responsiveness of these lymphocytes led us to the conclusion that the secondary lymphocytes obtained by stimulation with pokeweed mitogen (PWM)¹ represented a population of memory cells specific to PWM (9, 10).

Blast cells have also been obtained by sensitizing rat lymphocytes to xenogeneic or allogeneic fibroblast monolayers (11, 12). In this paper we show that these blasts, when removed from the foreign sensitizing monolayers and replated on syngeneic fibroblasts, transformed to secondary lymphocytes.

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¹ Abbreviations used in this paper: PBS, phosphate-buffered saline; PWM, pokeweed mitogen.

Analysis of the behavior of these lymphocytes suggests that they are specific memory cells.

Materials and Methods

Animals. Lymphocytes were obtained from Lewis rats. Mouse embryos for preparation of fibroblast monolayers were of strains C3H/eb, BALB/c, and C57BL/6, and rat embryos were of strains Lewis and Wistar.

Fibroblast Monolayers. Monolayers were prepared and maintained as previously described (13, 14). Monolayers for maintenance of growth and differentiation of blast cells and secondary lymphocytes were prepared by plating 5×10^6 fibroblasts onto 100-mm Falcon plastic Petri dishes (Falcon Plastics, Div. of BioQuest, Los Angeles, Calif.). They were used 3–5 days after preparation. Sensitizing monolayers were prepared by plating 3×10^6 fibroblasts onto 60-mm plastic Petri dishes. They were irradiated with 3,000 R 24 h later and then used. Target monolayers were prepared by plating 0.8×10^6 fibroblasts onto 35-mm plastic Petri dishes and irradiated with 3,000 R 24 h later. These monolayers were labeled with ^{51}Cr as follows: 1.5 μCi of [^{51}Cr]sodium chromate (Radiochemical Centre, Amersham, England) in 1.0-ml medium; 24 h later the medium was replaced by fresh medium and the monolayers were incubated for an additional 3–4 days before use.

Sensitization of Lymphocytes. Lewis rat lymphocytes were stimulated by xenogeneic (mouse) and allogeneic (Wistar rat) fibroblast monolayers. Suspensions of lymphocytes were prepared from lymph nodes as previously described (12). Lymph nodes were placed in phosphate-buffered saline (PBS) and forced through a wire mesh using a syringe plunger. The resulting suspensions were centrifuged and resuspended in Dulbecco's modified Eagle's medium containing 15% horse serum; 30×10^6 lymphocytes in 4-ml medium were plated on each sensitizing fibroblast monolayer. The cultures were incubated for 4 days at 37°C in a humidified incubator with a flow of 7% CO_2 in air. On the 3rd day the medium was replaced by fresh medium.

Growth of Blast Cells on Fibroblast Monolayers. By the 4th day when the cultures were rich in blast cells, the lymphoid cells were harvested, resuspended in fresh medium, and plated onto a rat Lewis fibroblast monolayer in a 100-mm Petri dish: 5×10^6 cells in 10-ml medium/dish. In order to obtain suspensions of secondary lymphocytes, these cultures were further incubated for 4–5 days.

Assay of Cell Transformation. After culturing on the fibroblast monolayer, the lymphoid cells were harvested from each plate, counted in a hemocytometer, spun down and resuspended in 2 ml of medium. 2 μCi of [^3H]thymidine (methyl-T, Radiochemical Centre, sp act 5 Ci/mM) in 0.2 ml PBS was added to each tube. The cells were incubated for 4 h at 37°C and then collected on Whatman GF/C filters. The filters were washed first with saline, then with 5% TCA, followed by absolute ethanol, and dried. Radioactivity was counted in 10-ml solution of: 0.4% wt/vol, 2,5 diphenyloxazole (PPO) and 0.005% wt/vol 1,4 bis-2-(4-methyl-5-phenyloxazolyl)benzene (POPOP) in toluene, using a Tri-carb scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

Target Cell Lysis. The extent of target cell lysis was assayed by the release of ^{51}Cr as previously described (14). Briefly, lymphoid cells in 1.5-ml culture medium were plated on ^{51}Cr -labeled target fibroblasts. The cultures were incubated at 37°C for the desired period (usually 42–48 h) and the amount of radioactivity released into the medium was measured as previously described (14). Radioactivity was counted in a well-type sodium iodide crystal Packard autogamma spectrometer (Packard Instrument Co., Inc.). Lysis is expressed as the percent of total radioactivity released into the medium, after correction for the spontaneous ^{51}Cr release in control plates. The standard deviation of triplicate plates did not exceed 2%.

Adherence of Lymphocytes to Target Monolayers. For assaying adherence, 5×10^6 secondary lymphocytes in 3 ml of medium were plated onto fibroblast monolayers in 60-mm Petri dishes. At intervals, the nonadhering lymphocytes were collected by pipetting and the monolayer was washed once with an additional 3 ml of medium which was combined with the first harvest. This represented the nonadherent fraction. The remaining monolayer and adhering lymphocytes were overlaid with a trypsin solution containing 0.005% neutral red and the plates were incubated for 30 min, after which the cell suspension was collected into a tube. This represented the adherent fraction. The neutral red was included to distinguish between fibroblasts and lymphoid cells (11). Lymphoid cells in both fractions were counted in a hemocytometer. Mean values were obtained from eight counts of each

sample. Adherence is expressed as percent of the total number of lymphoid cells obtained in the adherent fraction.

Results

Differentiation of Blast Cells to Secondary Lymphocytes. Lewis rat lymphocytes were sensitized to C3H, to BALB/c, or to Wistar rat fibroblasts by incubating them for 4 days on fibroblast monolayers. On the 4th day the lymphoid cells were harvested by pipetting and 5×10^6 cells were plated onto syngeneic (Lewis) fibroblasts. Fig. 1 shows the kinetics of thymidine uptake in

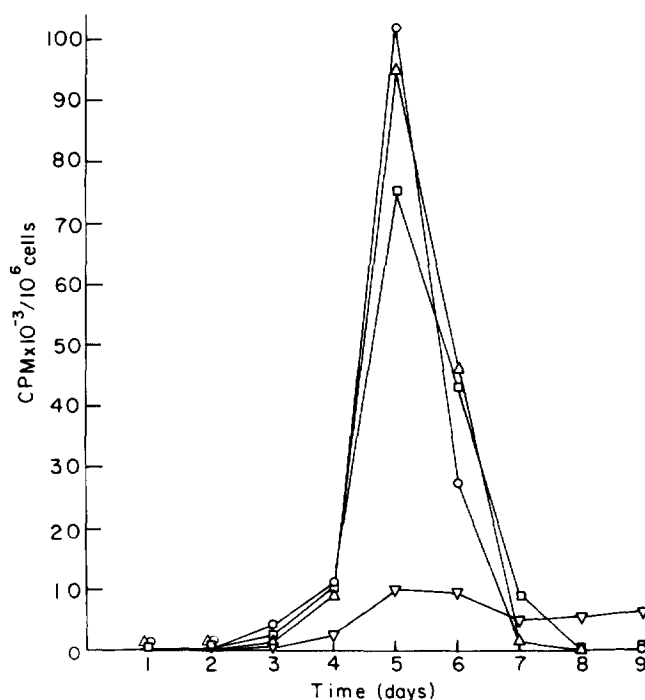


FIG. 1. Kinetics of [³H]thymidine uptake by rat lymphocytes sensitized against C3H (Δ—Δ), BALB/c (O—O), Wistar (rat), (□—□), or Lewis (syngeneic), (▽—▽) fibroblast monolayers. Lewis rat lymph node cells were incubated for 4 days on sensitizing monolayers. On the 4th day cells were harvested and replated on syngeneic Lewis fibroblast monolayers. Radioactivity was determined as described in the Materials and Methods.

cultures during sensitization on xenogeneic and allogeneic fibroblasts, and after transfer to syngeneic fibroblast monolayers. The percentages of blast cells in these cultures are shown in Fig. 2. 1 day after transfer from the sensitizing monolayers to syngeneic Lewis monolayers (day 5), the cultures were composed mostly of blast cells which showed a very high rate of thymidine uptake; on day 6, a decrease in both thymidine uptake and the percentage of blast cells was observed. On the 8th day and on subsequent days, the population was composed of small lymphoid cells (i.e., secondary lymphocytes) which did not incorporate thymidine. A similar pattern of transformation of blast cells to secondary

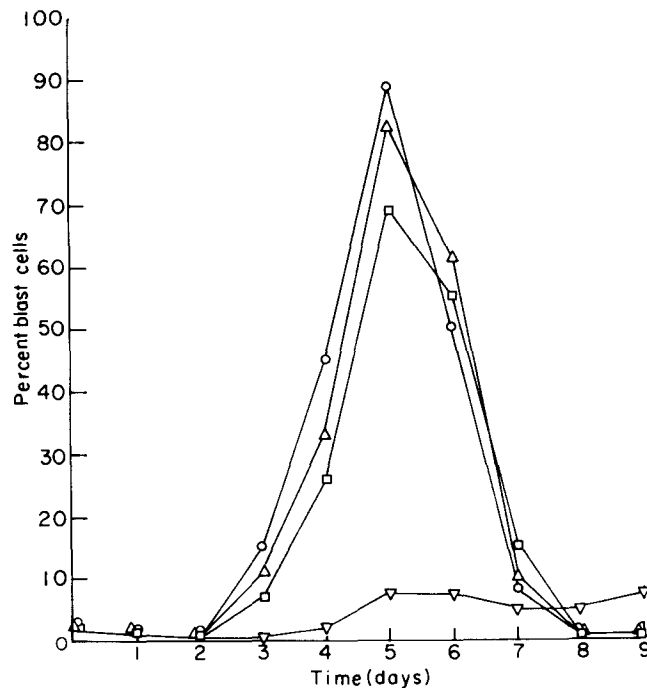


FIG. 2. Kinetics of morphological transformation of rat lymphocytes sensitized against C3H (Δ — Δ), BALB/c (O—O), Wistar (\square — \square), or Lewis (syngeneic) (∇ — ∇) fibroblast monolayers. Experimental conditions as described in Fig. 1. Percentage of blast cells was determined by differential counting in a hemocytometer.

lymphocytes has been demonstrated for blast cells induced by phyto mitogens (9). Proliferation of lymphocytes did not stop immediately after transfer from sensitizing to syngeneic fibroblast monolayers. Thus, after the stage of sensitization on the xenogeneic monolayer, proliferation of lymphocytes continues for an additional 24 h in absence of the specific sensitizing fibroblasts.

Restimulation of Secondary Lymphocytes by Specific Fibroblasts. To study the responsive potential of the secondary lymphocytes, 5×10^6 C3H-sensitized secondary lymphocytes were plated onto fibroblast monolayers, either identical with, or unrelated to the sensitizing fibroblasts. The transformation of lymphocytes in such cultures, as compared to normal lymphocytes, is shown in Fig. 3. In cultures of C3H monolayers seeded with normal lymphocytes, cell transformation began on the 3rd day of culture. On the other hand, in cultures of C3H monolayers seeded with secondary lymphocytes, thymidine uptake was already evident on the 1st day. No response was observed when secondary lymphocytes were exposed to third-party BALB/c or C57BL/6 fibroblasts (Fig. 4). Thus, secondary lymphocytes respond to the sensitizing antigen more promptly than do normal lymphocytes. A similar anamnestic response was observed when transformation of PWM-secondary lymphocytes in response to PWM was compared to that of normal lymphocytes (unpublished data).

Transformation of secondary lymphocytes was observed during the first 36 h. About this time toxic conditions began to develop, the cultures deteriorated, and

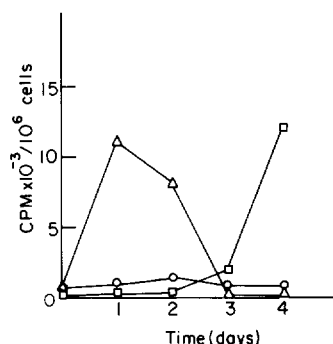


FIG. 3. [³H]thymidine uptake by C3H-sensitized (Δ—Δ), BALB/c-sensitized (O—O), and normal (□—□) rat lymphocytes incubated on C3H fibroblast monolayer.

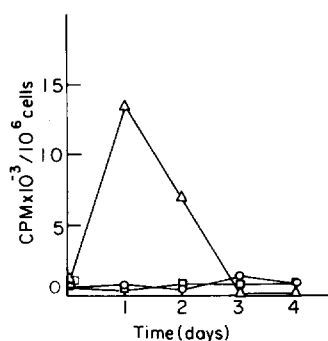


FIG. 4. [³H]thymidine uptake by C3H-sensitized secondary lymphocytes incubated on C3H (Δ—Δ), BALB/c (O—O), or C57BL/6 (□—□) fibroblast monolayers.

the lymphoid cell population died. This type of deterioration has previously been described in detail and termed "necrotic death" (9). Toxic conditions developed only in cultures in which secondary lymphocytes were plated on fibroblasts bearing the sensitizing antigens. When plated on unrelated fibroblasts, high viability was maintained. It should be noted that secondary lymphocytes showed no reactivity when cultured on fibroblasts antigenically unrelated to the primary sensitizing fibroblasts.

Target Cell Lysis by Secondary Lymphocytes. Blast cells obtained by primary sensitization of rat lymphocytes on mouse or rat fibroblast monolayers were able to lyse target fibroblasts specifically (11, 12). We therefore tested whether secondary lymphocytes, derived from sensitized blast cells, retained the capacity to lyse target cells. As shown in Table I, secondary lymphocytes did lyse target fibroblasts specifically. Lytic capacity was evident even after relatively long periods of incubation in culture (Table II). If medium was replaced every 3rd day and cells were passaged onto fresh syngeneic fibroblast monolayers every 7th–8th day, secondary lymphocytes could be maintained for at least 4 wk. Whenever the secondary lymphocytes were re-exposed to fibroblasts of the sensitizing strain, they lysed the target cells specifically. However, the lytic potency gradually diminished with time. A parallel decrease in transformation capacity was also observed (Table II).

TABLE I
*Specific Cytotoxicity of Rat Secondary Lymphocytes Sensitized
 against Mouse or Rat Fibroblasts**

Sensitizing fibroblasts	% ⁵¹ Cr release‡		
	Strain of target fibroblasts:		
	C3H	BALB/c	Wistar
C3H	48.8	15.3	7.2
BALB/c	8.5	45.1	4.2
Wistar	7.9	6.7	40.4

* Cytotoxicity was assayed 4 days after transfer of blast cells to syngeneic Lewis monolayers. At that time cultures were composed entirely of small lymphocytes. 4×10^6 lymphoid cells were plated on target monolayers.

‡ Determined after 48 h of incubation.

TABLE II
*Cytotoxicity and Transformation of C3H-Sensitized Rat
 Secondary Lymphocytes as a Function of Time after Sensitization*

Days after sensitization	⁵¹ Cr release from C3H target cells*	Thymidine uptake upon exposure to C3H monolayer‡
	%	
4§	50.3	ND
8	48.1	11,230
12	30.7	9,100
18	20.0	4,850
24	13.2	3,060

* Determined after 48 h of incubation.

‡ cpm/10⁶ cells. Determined after 24 h of incubation of lymphocytes on C3H monolayers. Values for lymphocytes incubated on syngeneic monolayers were 1,000–1,600 cpm/10⁶ cells.

§ Blast cells were plated directly on target monolayers without passage through the syngeneic monolayers.

|| 4 days after transfer of blasts to syngeneic monolayers, when cultures were composed entirely of small lymphocytes.

Adherence of Secondary Lymphocytes to Target Cells. It has been shown that in the graft reaction both the sensitization phase and the effector phase of target cell destruction involve contact between the lymphocytes and the target cells (15–18). We therefore tested the adsorption of secondary lymphocytes to the sensitizing fibroblasts. As seen in Tables III and IV, adherence occurs rapidly and is specific to the sensitizing antigen. Tables III and IV are representative of seven experiments with identical results. Although the differences in adherence to sensitizing and third-party monolayers were never large (12–19%), the difference was in the same direction in all experiments.

Tables V and VI show that the nonadherent fraction of the lymphocyte

TABLE III
*Kinetics of Adherence of C3H-Sensitized Rat Secondary Lymphocytes to Fibroblast Monolayers**

Time	% Adherence	
	Strain of fibroblasts:	
	C3H	Lewis
<i>h</i>		
1	21.4 ± 3.9	10.5 ± 2.1
2	25.3 ± 4.2	13.0 ± 3.0
4	28.9 ± 3.0	13.3 ± 0.9
6	30.6 ± 4.5	13.6 ± 2.2
10	24.8 ± 3.3	17.2 ± 1.7

* Adherence was assayed 4 days after transfer of blast cells to syngeneic Lewis monolayers.

TABLE IV
*Specificity of Adherence of Lewis Rat Secondary Lymphocytes Sensitized against Mouse or Rat Fibroblasts**

Sensitizing fibroblasts	% Adherence‡		
	Strain of test monolayer:		
	C3H	BALB/c	Wistar
C3H	30.4 ± 3.7	14.4 ± 0.8	10.2 ± 1.6
BALB/c	13.9 ± 1.9	29.7 ± 3.4	12.3 ± 1.2
Wistar	10.1 ± 0.8	12.0 ± 1.5	25.4 ± 2.9

* Adherence was assayed 4 days after transfer of blast cells to syngeneic monolayers.

‡ Determined after 4 h of incubation.

population was specifically depleted in the ability to lyse target cells. A parallel depletion in transformation capacity was also observed (Table VII). Nevertheless, the transformation and lytic ability were not entirely contained in the adherent fraction of the lymphocyte population.

Discussion

Small lymphocytes transform to blast cells (large pyroninophilic cells) in response to immunogenic stimulation by skin allografts. These blast cells then redifferentiate to secondary small lymphocytes which circulate and invade the graft (19). The processes of lymphocyte transformation in response to antigenic stimulation, followed by the reappearance of small lymphocytes, has been reproduced and analyzed in vitro in this study. Rat lymph node cells were cultured for 4 days on xenogeneic or allogeneic fibroblast monolayers. Transfor-

TABLE V
Cytotoxicity of Nonadherent C3H-Sensitized Secondary Lymphocytes*

Exp.	% ⁵¹ Cr release from C3H target monolayer‡		Relative decrease of cytotoxicity
	Adsorbing monolayer:		
	C3H	Lewis	%
1	28.1	42.5	34
2	8.1	26.1	69
3	19.6	36.7	47
4	13.4	25.2	47
5	25.0	35.9	30

* Lymphocytes were incubated on adsorbing monolayers for 4 h, the nonadherent cells were collected and plated (4×10^6 lymphoid cells) on C3H target monolayers.

‡ Determined after 48 h of incubation.

TABLE VI
Specific Reduction of Cytotoxicity in Nonadherent Lymphocytes*

Sensitizing fibroblasts	% ⁵¹ Cr release from target monolayer‡			
	Adsorbing monolayer:			
	C3H	BALB/c	Wistar	Lewis
C3H	24.9	39.0	40.1	42.2
BALB/c	31.0	20.7	34.6	35.0
Wistar	27.6	28.2	13.0	28.0

* Lymphocytes were incubated on adsorbing monolayers for 4 h, the nonadherent cells were collected and plated on target monolayers syngeneic to the sensitizing monolayer.

‡ Determined after 48 h of incubation.

mation of lymphocytes was first observed on the 3rd day of culture and reached a peak level on the 5th day. When transferred to syngeneic fibroblast monolayers, the blast cells reverted to small lymphocytes. This process was complete by the 4th or 5th day of culture on the syngeneic fibroblasts. The secondary lymphocytes seem to represent memory cells, and can be maintained for several weeks in vitro. As would be expected from memory cells, they manifest distinct immunospecificity: When returned to fibroblast monolayers of the primary sensitizing phenotype, the secondary lymphocytes undergo accelerated transformation. The capacity of the secondary small lymphocytes to thus retransform secondarily to blast cells gradually decreased with time. A concomitant decrease in the lytic potency of the cells was observed. These properties are similar to those observed

TABLE VII
*Transformation of Adherent and Nonadherent C3H-Sensitized
 Secondary Lymphocytes*

Fraction of lymphocytes	Adsorbing monolayer	cpm/10 ⁶ cells*
Unadsorbed Nonadherent‡	None	16640 ± 1,171
	C3H	8018 ± 791
	Lewis	15793 ± 920
Adherent§	C3H	22832 ± 1,024

* Thymidine uptake was determined after 30 h of incubation on C3H monolayers. Values for lymphocytes incubated on syngeneic Lewis fibroblasts were 3,000–3,500 cpm/10⁶ cells.

‡ Lymphocytes were incubated on adsorbing monolayers for 4 h, the nonadherent cells were collected and plated (4 × 10⁶ lymphoid cells) on C3H monolayers.

§ The nonadherent cells were removed, fresh medium was added, and the cultures were further incubated.

in anamnestic responses *in vivo* (20) and to those manifested *in vitro* by *in vivo* sensitized cells, which undergo rapid transformation when exposed to the immunogen, and are capable of lysing target cells (4, 21). The accelerated response *in vitro* of lymphocytes sensitized *in vivo* also persists for a certain time after immunization and then disappears (4). It should be noted, however, that the ability to mediate second set graft rejection *in vivo* persists for a significantly longer time than the reactivity of memory cells produced *in vitro*. This could be attributed either to the culture conditions, which shorten the life span of cells, or to the possibility that *in vitro*, the relatively short exposure to the immunogen was not sufficient to induce differentiation of long-lived memory cells. Secondary lymphocytes, capable of both accelerated blast transformation and target cell killing, are progeny of the blast cells obtained by primary sensitization, since blast cells fractionated on a bovine serum albumin density gradient reverted to small lymphocytes capable of both functions (unpublished data). Whether both functions are mediated by the same or different cells is not known.

In previous papers we described the development *in vitro* of highly enriched populations of secondary ("memory") lymphocytes with specific receptors for PWM (9, 10). Over 80% of the PWM-secondary lymphocytes adhered to fibroblast monolayers to which PWM has been conjugated as compared to 10–20% adherence of normal lymphocytes. In the present study we have found that secondary lymphocytes produced after sensitization on xenogeneic or allogeneic fibroblasts manifested specific adherence, but the extent of adherence was significantly lower than that observed with PWM-induced secondary lymphocytes. This may be attributed either to differences in the density of antigenic determinants on the fibroblast (the density of conjugated PWM determinants being higher than that of the strain specific surface antigens), or to differences in the density and/or affinity of the receptors for antigens.

Lymphocytes sensitized against fibroblast monolayers *in vitro* are immunoreactive *in vivo*. They have been shown to cause accelerated skin graft

rejection and to be highly effective in inhibiting the growth of concomitantly injected tumor cells (22, 23). Hence, *in vitro* sensitization is an effective means of inducing transplantation immunity. The finding that *in vitro* sensitized lymphocytes differentiate to memory cells which can be maintained in culture suggests that the system described can serve as a model for studying immunological memory in transplantation immunity.

Summary

The *in vitro* generation of memory cells reactive to transplantation antigens is described. Blast cells, obtained from rat lymphocytes sensitized on xenogeneic or allogeneic fibroblast monolayers, reverted to secondary small lymphocytes after transfer from the foreign sensitizing to syngeneic monolayers. These secondary small lymphocytes had a limited *in vitro* life span of 4–6 wk. They manifested properties of memory cells: upon re-exposure to fibroblasts of the sensitizing phenotype, the secondary lymphocytes adhered to the fibroblast monolayer and transformed into blast cells with cytotoxic activity. The response of secondary lymphocytes was rapid, compared to that of normal lymphocytes, and directed specifically against the primary sensitizing antigens.

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