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Failure of Ovalbumin Associated with Allogeneic Spleen Cells to Stimulate Proliferation of Lymph Node Cells of Immune Mice[1] (39339)

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The specificity of cytotoxic T-cells for antigen modified target cells has been shown to include the H-2 antigen of the cell used to stimulate activation of the effector cells (1-6). Using the incorporation of [^{3}H]-thymidine as an assay of lymphocyte blastogenesis, Rosenthal and Shevach have shown that in guinea pigs proliferation of immune lymphocytes induced by PPD bound to macrophages was much greater if the macrophages were syngeneic to the responding cells than if they were allogeneic (7). A similar requirement of syngeneic macrophages has been more difficult to demonstrate in mice because of the inhibitory effect of mouse macrophages on cell proliferation and because of the higher background produced by a much more prompt mixed lymphocyte reaction in this specie.

In the experiments reported here we have partially circumvented the inhibitory effect of mouse macrophages by reducing their number and largely eliminated the mixed lymphocyte reaction by using F_1 mice made chimeric with bone marrow from one of the parental strains. Using lymph node cells of immunized chimeras we have shown that the proliferative response to a thymus-dependent antigen, ovalbumin (OVA), is much greater when the antigen is presented on cells of the parental strain used for the bone marrow injection than on cells of the other parental strain.

Materials and methods. Animals. BALB/c, A/J, CAF_1 (BALB/c × A/J), and LAF_1 mice were purchased from Jackson Laboratories (Bar Harbor, Maine).

Immunization. Mice were immunized with 400 μg of OVA in complete Freund's adjuvant (CFA) in the footpad.

Pulsing of spleen cells with antigen. The cells used were normal spleen cells which were incubated at 37° for 7 hr in a petri dish, and the nonadherent cells were transferred to a separate dish. The nonadherent cells and in one experiment the adherent cells were then incubated for 5 hr with or without 10 or 100 μg OVA ml 4×10^6 cells. Preliminary experiments demonstrated the requirement for 5-hr incubation with antigen to yield maximum stimulation. The cells were then washed three times to remove excess antigen. The washed spleen cells were then mixed with lymph node cells from mice immunized in the footpad 2 to 3 weeks earlier. In some cases 25 μg of mitomycin C was added to the spleen cells for the last 3 min of incubation. In one experiment the spleen cells were heated to 56° for 30 min after washing, a procedure found to produce nearly 100% loss of ability to exclude dye.

Culture conditions. Culture conditions were similar to those previously described (8). Two million immune lymph node cells plus 2×10^6 spleen cells (control or pulsed) were cultured at 37° in 1 ml of RPMI-1640 medium tubes in a humidified CO_2 incubator (5% CO_2 and 95% air). The medium contained 5% heat-inactivated rat serum, 100 units of penicillin G, and 100 μg of streptomycin/ml. After 3 days of culture 1 μCi of tritiated thymidine (T-^{3}H) (6 Ci/mmole) was added for the final 5 hr and DNA precipitated on glass fiber filters (Whatman GF/C grade) with trichloracetic acid (TCA) and counted in a liquid scintillation counter.

Labeling ovalbumin with ^{125}I. To 5 mCi carrier-free ^{125}I in 0.1 ml was added in sequence 0.05 ml of 0.01 *N* KI, 0.5 *N* HCl dropwise until acid, 0.05 ml of 0.1 *N* $NaNO_2$, 0.05 ml of 0.1 *M* urea, and 5 mg of OVA in 0.5 ml 0.8% NaCl solution (cf. refs. 9 and 10). The mixture was immediately brought to pH 11 by dropwise addition

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of 0.2 *M* sodium carbonate buffer (pH 12) and as soon as iodine color disappeared, was brought back to neutrality by dropwise addition of 0.1 HCl. The labeled OVA was then dialyzed at 4° for 4 days against dilute KI solution with daily changes. The resulting ^{125}I-OVA contained 80,000 counts per μg (Folin) and 98% of the radioactivity was precipitable by TCA.

Preparation of chimera. CAF_1 mice were lethally irradiated (800 R of ^{60}Co) and reconstituted with 10×10^6 BALB/c bone marrow cells. A test for chimerism was made after 4 weeks on spleen cells taken from the reconstituted mice and separated twice on glass-bead columns (11). These separated spleen cells, which are mostly T-cells, were incubated with BALB/c anti A serum prepared according to the method of Miller and Mitchell (12), washed, and then mixed first with rabbit anti-mouse immunoglobulin serum and then with goat anti-rabbit immunoglobulin serum labeled with fluorescein. Twelve percent of splenic T-cells from the reconstituted mice were labeled by this technique compared to 53% of splenic T-cells from normal CAF_1 mice and 5% of splenic T-cells from BALB/c mice. This indicated that the majority of splenic T-cells were donor BALB/c type. The chimeras were then immunized with OVA in CFA in the footpad. Two to three weeks later, cells from their lymph nodes were removed and cultured with BALB/c or A/J spleen cells in the regular manner.

Results. Stimulatory capacity of normal spleen cells. Mice were immunized with OVA in CFA in the footpad. Two to three weeks postimmunization, single cell suspensions of the popliteal and mesenteric lymph nodes were cultured with nonadherent spleen cells previously incubated with antigen. The results of two such experiments are shown in Table I and demonstrate the excellent stimulatory capacity of the pulsed spleen cells in the mouse system. The amount of radioactivity incorporated into the lymph node cells stimulated by antigen-pulsed spleen cells (group Ic) was nearly as great as when 100 μg of antigen were left in the culture for 3 days (group Ib). In Experiment II, the OVA used for coating the normal spleen cells was labeled with ^{125}I. After three washings the amount of ^{125}I-labeled OVA that remained with the spleen cells was 0.1 μg. This amount of cell-bound OVA was almost as effective (89%) as 100 μg of free OVA in stimulating lymph node

TABLE I. THE RESPONSE OF LYMPH NODE CELLS FROM IMMUNIZED MICE TO ANTIGEN-PULSED SPLEEN CELLS TREATED WITH MITOMYCIN C AND HEAT.[a, b]

Group	Antigen in 5-hr spleen cell culture (μg)[c]	Treatment of spleen cells after 5-hr culture[d]	Antigen in 3-day mixed culture (μg)	cpm ± SE	
				Expt. 1	Expt. 2
I a	None	None	None	5,400 ± 70	5,200 ± 100
b	None	None	100	26,000 ± 1,000	25,400 ± 1,800
c	100	None	None	20,100 ± 900	23,300 ± 300
d	100	None	100	33,200 ± 2,700	27,500 ± 1,300
II a	None	Mit C	None	1,400 ± 200	1,000 ± 90
b	None	Mit C	100	12,000 ± 500	10,500 ± 450
c	100	Mit C	None	9,300 ± 900	9,300 ± 500
d	100	Mit C	100	14,600 ± 300	15,000 ± 800
III a	None	Heat	None	2,700 ± 400	–
b	None	Heat	100	15,600 ± 400	–
c	100	Heat	None	2,500 ± 80	–
d	100	Heat	100	15,700 ± 2,600	–

[a] BALB/c mice were immunized in the footpad with 400 μg of OVA in CFA and after 2 to 3 weeks popliteal lymph nodes were removed and cell suspensions prepared.

[b] Normal BALB/c spleen cells were incubated for 1 hr at 37° in Falcon plastic culture petri dishes to remove adherent cells. The nonadherent cells were cultured for 5 hr at 37° with or without OVA. The cells were then spun down and washed twice. Spleen cells 2×10^6 were then incubated with 2×10^6 immune lymph node cells for 3 days, with 1 μCi of ^{3}H-T added for the last 5 hr.

[c] In Experiment II, the OVA added to spleen cells was labeled with ^{125}I.

[d] Spleen cells were washed three times (None), treated with 25 μg of mitomycin C for the final half-hour of 5-hr culture, and washed three times (Mit C), or were washed three times and then heated at 56° for 30 min (Heat), before they were mixed with immune lymph node cells.

cells. In other experiments the addition of 10 μg of free OVA left in the 3-day culture was only 23% stimulatory as 100 μg (data not given).

Effect of treating spleen cells with mitomycin C or heat. The results in Table I (group II) show that mitomycin C treatment of the spleen cells at the end of the 5-hr culture reduced the net counts incorporated as a result of the antigen added but increased the ratio of experimental to control counts. When the antigen-pulsed spleen cells were heated at 56° for 30 min before addition of lymph node cells, however, they completely lost the ability to stimulate (group IIIc). This indicates that the antigen remaining with the cells was insufficient to stimulate unless bound to a living cell. The heated cells were not inhibitory to a response to antigen added to the 3-day culture (groups IIIb and d).

Failure of BALB/c lymph node cells to respond to antigen on allogeneic LAF_1 spleen cells. Spleen cells from syngeneic [BALB/c as well as allogeneic (LAF_1)] mice were pulsed with antigen and mixed with BALB/c immune lymph node cells. The results from these experiments given in Table II show that antigen associated with LAF_1 spleen cells was no more stimulatory to BALB/c immune lymph node cells than LAF_1 spleen cells alone. The incremental effect of adding antigen to the mixed culture was clearly present, however. Because of the high background of stimulation from the mixed lymphocyte reaction we made chimeras by lethally irradiating CAF_1 mice and reconstituting them with BALB/c bone marrow cells. The BALB/c cells which repopulated the lymph nodes of these chimeras were presumably tolerant to A/J antigens because they responded only slightly in a mixed lymphocyte reaction. Chimeras thus prepared were immunized with OVA and their lymph node cells cultured as before with OVA-pulsed spleen cells from BALB/c and A/J mice. The response to OVA of these chimeras was lower than with normal mice, but the results shown in Table III confirm the requirement that antigen be presented on syngeneic spleen cells. Antigen associated with BALB/c spleen cells was considerably more stimulatory than antigen associated with A/J spleen cells. The slight stimulation by the latter may have been due to incomplete substitution in the chimeras.

Discussion. One-tenth microgram of OVA bound to cells was almost as effective as 100 μg of free OVA in stimulating a proliferative response of lymph node cells from immunized mice. The relatively greater efficiency of bound as compared to free antigen may be much higher than these results indicate since most of the cell associated antigen may have been irrelevant (i.e., in the wrong place or on the wrong cell), and the "free" antigen probably becomes "bound" when it is added to the culture. There is now considerable reason to doubt that free antigen is recognized by T-cell directly since T-cells are not absorbed by antigen columns (13) and the specificity of the T-cell for antigen appears to always include

TABLE II. FAILURE OF BALB/c LYMPH NODE CELLS TO RESPOND TO ANTIGEN ON ALLOGENEIC SPLEEN CELLS.

Strain source of spleen cells	Antigen in 5-hr culture of spleen cells (μg)	Antigen in 3-day mixed culture (μg)	cpm ± SE	
			Expt. 1	Expt. 2
BALB/c	None	None	750 ± 60	700 ± 40
BALB/c	None	100	5,700 ± 700	5,000 ± 200
BALB/c	100	None	3,600 ± 200	3,000 ± 25
BALB/c	100	100	7,300 ± 40	7,400 ± 600
LAF_1	None	None	20,900 ± 1,500	28,200 ± 2,100
LAF_1	None	100	27,100 ± 1,000	34,300 ± 2,500
LAF_1	100	None	20,600 ± 1,800	21,400 ± 150
LAF_1	100	100	26,200 ± 1,400	32,000 ± 2,800
Net response to antigen on BALB/c cells			2,850	2,300
Net response to antigen on LAF_1 cells			−300	−6,800
Net response to free antigen in presence of BALB/c cells			4,900	4,300
Net response to free antigen in presence of LAF_1 cells			6,200	6,100

TABLE III. RESPONSE OF LYMPH NODE CELLS FROM IMMUNIZED BALB/c TO CAF_1 CHIMERAS TO ANTIGEN ON PARENTAL SPLEEN CELLS.[a]

Experiment	Source of spleen cells	cpm ± SE when spleen cells[b] cultured			
		Without antigen	with antigen	Net cpm	Ratio
I	BALB/c	1,200 ± 83	3,800 ± 100	2,600	3.2
	A/J	3,100 ± 80	4,900 ± 200	1,800	1.6
II	BALB/c	700 ± 26	2,600 ± 400	1,900	3.6
	A/J	2,000 ± 100	2,100 ± 20	100	1.0
III	BALB/c	500 ± 80	2,500 ± 400	2,000	5.0
	A/J	1,300 ± 0	1,700 ± 65	400	1.3
IV	BALB/c	700 ± 300	2,000 ± 100	1,300	2.9
	A/J	800 ± 47	1,000 ± 50	200	1.2

[a] Chimeras were prepared by injecting BALB/c bone marrow cells into lethally irradiated CAF_1 mice. Four weeks later the chimeras were immunized with OVA in the footpad. Lymph node cells from these mice were later cultured with BALB/c or A/J spleen cells preincubated with or without 100 μg of OVA.

[b] Spleen cells were prepared as in Table I, except in Expt. III where the adherent cells were used.

the H-2 antigen of the presenting cell (1–7). This may be true of non-H-2 intrisic cell surface antigens as well as of extrinsic protein antigens such as OVA (6, 7). It is difficult to understand why there should be such an inclusion of H-2 specificity if non-H-2 antigens could be recognized by T-cells directly.

The present experiments illustrate a possible method of investigating the specificity of T-cells and answering the questions discussed above. Since the only parental cells in the chimeras we produced were bone marrow-derived cells, the specificity of the responding cells for antigen presented on cells of the parental strain injected implies that under *in vivo* immunizing conditions, antigen is presented to the T-cell by a bone marrow-derived cell. An important question that may be answerable by these techniques is whether BALB/c T-cells would respond to antigen presented on A/J cells if they are first sensitized by antigen on A/J cells. We would predict that if sensitization can be accomplished by antigen-pulsed allogeneic cells, the T-cells of such an animal would respond better to antigen presented on allogeneic cells of the same strain than to antigen presented on syngeneic cells.

These experiments do not answer the question which bone marrow-derived cell or cells (B, T, or monocyte) is responsible for presenting the antigen.

It is possible as proposed by Rosenthal and Shevach (7) and Greineder and Rosenthal (14) that the macrophage is the essential cell in both antigen presentation and stimulation in mixed lymphocyte reactions. In experiments with mouse cells, small numbers of macrophages were highly stimulatory to allogeneic cells with which they were in contact for only a few hours (11, 15). Thus the nonadherent spleen cells used in these experiments may have been effective in presenting antigen because they contained only the minimum number of macrophages required.

Summary. Ovalbumin-pulsed spleen cells were found to stimulate thymidine uptake of lymph node cells of syngeneic mice immunized with ovalbumin in complete Freund's adjuvant after treatment of spleen cells with Mitomycin C but not after heating the spleen cells at 56° for 30 min. Ovalbumin-pulsed spleen cells of allogeneic mice failed to stimulate the immune lymph node cells more than unpulsed cells, although a net increase in the thymidine uptake above the allogeneic stimulation was observed when free ovalbumin was added to the mixed culture. To eliminate the high background of the mixed lymphocyte reaction, F_1 mice were made chimeric with bone marrow of one of the parental strains. Using lymph node cells of the immunized chimeras, the stimulation by pulsed spleen cells was much greater when antigen was presented on cells of the parental strain used for bone marrow injection than when presented on cells of the other parental strain.

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