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# Activation of T-Lymphocye Helper Function by Brief Exposure to Antigen-Pulsed Macrophages[1] (39126)

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The role of macrophages in the induction of an immune response *in vitro* has been the subject of much research in recent years (1). In spite of this, little is known of the functional role of the macrophage in the inductive process. Using the guinea pig, Rosenthal and coworkers have shown that the presence of macrophages appears to be necessary for the antigen-induced proliferation of immune lymphocytes, and that a brief exposure of macrophages to antigen, prior to the addition of immune lymphocytes, is as effective a means of stimulation by antigen as the continued presence of antigen in the macrophage–lymphocyte culture (2). In addition, Rosenthal *et al.* have shown that macrophages must be metabolically active (3, 4), and that the macrophages and lymphocytes must be histocompatible (5) for effective lymphocyte activation by antigen. Greineder and Rosenthal have also shown that guinea pig macrophages are effective stimulators of allogeneic lymphocytes in a mixed lymphocyte culture (MLC)[2] (6). Talmage *et al.* similarly have shown that in the MLC using mice, allogeneic macrophages are highly stimulatory and elicit a maximum response if left in contact with the responding cells for only a few hours (7). In this paper, we report on the ability of macrophage-associated antigen to stimulate the activation of immune T-lymphocytes to produce nonspecific helper activity for antibody production.

*Materials and methods. Mice.* Male $LAF_1$ and BALB/c mice were obtained from Jackson Laboratory, Bar Harbor, Maine. $LAF_1$ mice were injected ip with 2 mg ovalbumin (OVA, Miles Laboratory, Kankakee, Ill.) in complete Freund's adjuvant (Colorado Serum Co., Denver, CO.) and sacrificed 1 to 2 months later.

*Column purified ovalbumin-immune spleen cells (OVA-ISC).* Columns (1 × 20 cm) were filled with glass beads (Superbrite, 3M Co., St. Paul, Minn.) and equilibrated with Eagle's Minimum Essential Medium (Colorado Serum Co., Denver, CO.) containing 10% fetal calf serum (Grand Island Biochemical Co., Grand Island, Nebraska) (MEM). Spleen cells from OVA-immune mice were applied in a small volume and moved into the upper half of the column. After incubation at 37° for 30 min, the cells were moved to the lower half of the column and incubated an additional 30 min. The nonadherent cells were eluted in 15 ml of medium and resuspended to a concentration of $2 \times 10^6$ cells/ml.

*Ovalbumin-pulsed macrophages.* Uninduced mouse peritoneal exudate cells ($5 \times 10^5$), obtained by washing the peritoneal cavity, were incubated in 35 mm plastic Petri dishes (Falcon Plastics, Oxnard, Calif.) at 37° with 5% $CO_2$ for 1 hr. The cells were washed three times to remove nonadherent cells. OVA (200 μgs) in 0.5 ml MEM was added to each dish and the cells were incubated at 37° for an additional hour. The antigen-pulsed macrophages were washed three times with MEM to remove unbound OVA. Control macrophage cultures were incubated in the absence of OVA.

The peritoneal exudate cells contained about 70% typical macrophages. After the two 1-hr incubations, about 10% of the cells were removed by washing. Most of the cells removed were lymphocytes. The 4-hr incuba-

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[2] Abbreviations used: MLC, mixed lymphocyte culture; OVA, ovalbumin; OVA-ISC, ova-immune spleen cells; MEM, Eagle's minimal essential medium + 10% fetal calf serum; NSC, nonimmune spleen cells; SRBC, sheep red blood cells; and PFC, plaque-forming cells.



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tion of the ovalbumin-pulsed macrophages with OVA-ISC caused the additional release of approximately 15% of the original peritoneal exudate cells. After 24 hr of incubation, approximately 65% of the cells remained on the dishes, 99% of which were macrophages.

*Culture.* The column-purified OVA-ISC ($2 \times 10^6$) were added to ovalbumin-pulsed macrophages and incubated with rocking at 37° and 5% of $CO_2$ for various lengths of time. At timed intervals, the nonadherent cells were removed with a pipette and placed in 35 mm culture dishes containing nonimmune spleen cells ($3 \times 10^6$, NSC) and sheep red blood cells (SRBC). The number of direct plaque-forming cells (PFC) to SRBC was determined after 4 days of Mishell–Dutton type culture (8) by the Jerne plaque assay (9).

*Results and discussion.* Table I shows the effect of culturing $5 \times 10^6$ irradiated OVA-ISC and $10^6$ NSC in the presence of SRBC and varying amounts of OVA. The optimum amount of OVA was 10 μg added to the culture. The effect of 1 μg was only 10% of the optimum and 0.1 μg had no demonstrable effect.

Since Talmage *et al.* (7) have recently shown that mouse T-lymphocytes in MLC could be activated by brief exposure to allogeneic macrophages, it was of interest to determine if the production of T-cell helper activity for an antibody response could be induced by a similarly brief exposure to antigen-pulsed macrophages. As a source of immune T-cells, we used glass bead column-passed OVA-ISC. Column-passed spleen cells have previously been shown to be enriched for T-cells while depleted of macrophages and B-cells (10). After incubation with OVA-pulsed macrophages, these activated T-cells were mixed with NSC, which provided antibody-producing B-lymphocytes and SRBC and the antibody response to SRBC was measured several days later. Any increase in the anti-SRBC response would then measure the extent of T-cell helper activity for antibody production.

TABLE I. EFFECT OF OVALBUMIN-INDUCED HELP ON THE SHEEP RBC RESPONSE

| OVA added (μg)[a] | PFC/culture[b] |
|---|---|
| None | 13 ± 6 |
| 0.1 | 3 ± 3 |
| 1 | 67 ± 21 |
| 10 | 620 ± 110 |
| 100 | 173 ± 45 |

[a] Ovalbumin immune spleen cells (OVA-ISC) were obtained from OVA immune mice given 750 R $^{60}Co$ irradiation immediately before sacrifice. $5 \times 10^6$ OVA-ISC thus obtained were mixed with $10^6$ normal spleen cells, SRBC, and varying amounts of OVA and cultured for 4 days in a Mishell–Dutton rocking culture.

[b] Each determination represents the mean of triplicate cultures ± standard error.

TABLE II. STIMULATION OF IMMUNE T-LYMPHOCYTE HELPER FUNCTION BY BRIEF EXPOSURE TO OVALBUMIN-PULSED MACROPHAGES

| $3 \times 10^6$ NSC | Addition[a] | PFC/culture[b] |
|---|---|---|
| + | None | 15 ± 6 |
| − | Adherent | 10 ± 0 |
| + | Adherent | 10 ± 2 |
| − | Nonadherent | 210 ± 58 |
| + | Nonadherent | 1275 ± 41 |

[a] Column-passed ovalbumin-immune spleen cells (OVA-ISC, $2 \times 10^6$) were incubated for 4 hr with ovalbumin-pulsed macrophages. The adherent and nonadherent cells were then separated and cultured for 4 days in Mishell–Dutton type culture with or without added normal spleen cells (NSC, $3 \times 10^6$) as indicated. All cultures contained sheep erythrocytes (SRBC) and the direct plaque-forming cells (PFC) to SRBC were determined after 4 days of *in vitro* culture.

[b] Same as Table I.

As shown in the initial experiment (Table II), the immune T-cells were capable of being activated for helper function by only a brief 4-hr exposure to the OVA-pulsed macrophages. The addition of NSC to the nonadherent activated T-cells resulted in an almost 100-fold increase in PFC as compared to NSC alone (1275 vs 15). A separate culture of activated T-cells that are deficient in B-cells, produced more PFC (210) than did NSC. The failure of the adherent cells after exposure to immune T-cells to increase the PFC response of NSC suggests that few

T-cells remained adherent to the macrophages or that the macrophages produced a suppressive effect. These results also suggest that T-cell activation for helper function can be effected by only brief exposure to antigen-pulsed macrophages and that the cellular interaction does not require long-term contact since most of the activated T-cells appear to be easily removed from the macrophages.

The optimal time for the OVA pulse was not determined, but previous work (11) has shown that optimal immunogenic activity occurs with an antigen–macrophage pulse of 15 to 30 min. Using $^{125}$I-labeled OVA (12), it was found that macrophages retained, after washing, less than 1% of a 100 $\mu$g pulse of OVA. This is less than that required to give an optimal PFC response when added to the culture for 4 days.

The kinetics of this T-cell activation show that optimal helper function occurred after a 4-hr incubation with the OVA-pulsed macrophages (Fig. 1). Less helper function was detected after 2 or 8 hr of contact, and almost no activation occurred after a 0- or 24-hr contact. This observation also suggests that carryover of antigen or antigen-pulsed cells is not responsible for T-cell activation since such carryover could presumably occur at all times of T-cell transfer and result in a similar extent of activation. It seems likely, therefore, that T-cell activation results from a relatively short period of recognition and interaction with antigen-pulsed macrophages.

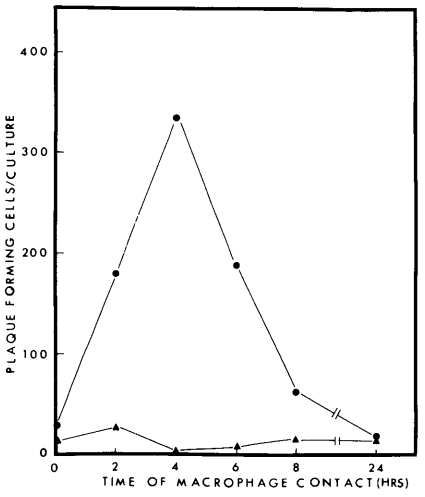


FIG. 1. Time course of immune T-lymphocyte activation by ovalbumin-pulsed macrophages. Column-passed OVA-ISC ($2 \times 10^6$) were incubated with OVA-pulsed macrophages for various lengths of time as indicated in the abscissa. The nonadherent cells were removed at each timed interval and placed in Mishell–Dutton type culture with SRBC and NSC ($3 \times 10^6$), and the number of PFC was determined after 4 days of culture (circles). The number of PFC from NSC cultured alone with SRBC was also determined at each timed interval (triangles).

The fact that a 4-hr contact between antigen-pulsed macrophage and T-cell is optimal for T-cell activation is puzzling. The decline in activity after 4 hr of incubation may be due either to macrophage toxicity or to the transfer of suppressor cells, which may require longer than helper cells to become activated or to detach from the culture dishes.

In addition to recognizing antigen-pulsed macrophages, T-cell activation also requires that the cellular interaction occurs under conditions allowing for optimal metabolic activity (Table III). Good helper activity was found if the T-cell–macrophage contact occurred at 37° (648 PFC) but not at 4° (123 PFC). This 4° incubation had no deleterious effect on the T-cells since the readdition of soluble OVA after the 4° incubation resulted in a similar number of PFC (648 PFC) as T-cells incubated with macrophage at 37°C. This experiment also demonstrated that a 4-hr contact with OVA-pulsed macrophages resulted in as efficient T-cell activation as having 100 $\mu$g of soluble OVA present in culture with the T-cells continuously for 4 days. It was also found that the antigen-pulsing of the macrophages was temperature-dependent and was more effective at 37° than at 4°. The diminished ability of macrophages pulsed with OVA at 4° to activate T-cells may be due to the decreased ability of macrophages to bind antigen at this temperature as shown previously (11), or to a requirement for metabolic activity in the macrophage.

Since Rosenthal and Rosenstreich (5) have shown that in guinea pigs optimal T-cell activation occurred only when the antigen-pulsed macrophages and immune T-cells were syngeneic, it was important to deter-

mine if the activation for helper function in our system was also restricted to a similar histocompatibility. Therefore, antigen-pulsed or untreated syngeneic $LAF_1$ or allogeneic BALB/c macrophages were compared in their ability to activate immune T-cells for helper function during a 4-hr contact (Table IV). OVA-pulsed $LAF_1$ macrophages showed a much greater ability to activate immune $LAF_1$ T-cells for helper function for a SRBC response than the corresponding untreated macrophages (608 PFC vs 88 PFC). This result provides further support for the assumption that the T-cells recognize the antigen in association with macrophages, but are not activated by recognition of macrophages alone.

The low level of activation produced by macrophages not pulsed with OVA may represent a primary response to antigens found in fetal calf serum. In contrast, immune $LAF_1$ T-cells were activated for helper function by allogeneic BALB/c macrophages to almost the same extent regardless of the OVA pulse. These results suggest that immune T-cells may be specifically activated to helper function by the recognition of antigen in association with syngeneic histocompatibility antigens on the macrophage surface. Thus, the T-cell may recognize only the antigen-altered surface of syngeneic macrophages according to the "self + *X*" hypothesis proposed by Lawrence (13) or the "self − *X*" hypothesis of Talmage and Hemmingsen (7). However, these results are not conclusive because the BALB/c macrophages induce some helper function in the absence of OVA.

The results we have reported here are consistent with the hypothesis that T-cell activation requires two distinct events (14). The first event is the recognition process in which T-cells come into physical contact with the macrophage. Our results suggest that this recognition process involves more than the antigenic determinants alone and requires that these determinants be presented in conjunction with self components presented on syngeneic, but not allogeneic, macrophage surfaces. This recognition process alone, however, is not sufficient for T-cell activation since the activation requires that the interacting cells be metabolically active.

One possibility is that the initial antigen-induced cell interaction between T-cells and

TABLE III. TEMPERATURE DEPENDENCE OF MACROPHAGE-INDUCED IMMUNE T-LYMPHOCYTE ACTIVATION

| $3 \times 10^6$ NSC | OVA pulse (°C)[a] | Contact[b] | PFC/culture[c] |
|---|---|---|---|
| +[d] | − | − | 87 ± 3 |
| − | 37 | 37 | 17 ± 3 |
| + | 37 | 37 | 648 ± 47 |
| + | 37 | 4 | 123 ± 78 |
| + | 37 | 4[e] | 648 ± 105 |
| + | 4 | 37 | 310 ± 68 |
| + | 4 | 4 | 210 ± 12 |

[a] Macrophages were pulsed with 100 μg of ovalbumin for 1 hr at the indicated temperature.

[b] Column passed OVA-ISC were incubated for 4 hr with OVA-pulsed macrophages at the indicated temperature. The nonadherent cells were removed and incubated in Mishell-Dutton type culture with or without NSC.

[c] Same as in Table I, Footnote *b*.

[d] Column-passed OVA-ISC were incubated for 4 hr in dishes without macrophages. The nonadherent cells were removed and added to the NSC.

[e] Soluble OVA (100 μg) was added to these cultures after the nonadherent cells were removed from the macrophages and added to the NSC.

TABLE IV. IMMUNE T-LYMPHOCYTE ACTIVATION BY SYNGENEIC AND ALLOGENEIC OVALBUMIN-PULSED MACROPHAGES

| $3 \times 10^6$ NSC | Macrophage source | Ovalbumin pulse[a] | PFC/culture[b] Expt 1 | PFC/culture[b] Expt 2 |
|---|---|---|---|---|
| +[c] | − | − | 15 ± 6 | 5 ± 3 |
| + | $LAF_1$ | − | 5 ± 0 | 88 ± 42 |
| + | $LAF_1$ | + | 108 ± 9 | 608 ± 133 |
| + | BALB/c | − | 58 ± 16 | 343 ± 112 |
| + | BALB/c | + | 33 ± 17 | 597 ± 86 |

[a] Column-passed $LAF_1$ OVA-ISC were incubated with $LAF_1$ or BALB/c macrophages, untreated or pulsed with OVA as indicated. The nonadherent cells were removed and incubated in Mishell-Dutton type culture with NSC and SRBC for 4 days, and the number of PFC was determined.

[b] Same as in Table I, Footnote *b*.

[c] Same as in Table II, Footnote *d*.

macrophages activates the macrophages which in turn activate the T-cells to produce helper function for antibody production. This T-cell activation process seems to be a relatively rapid event and does not require prolonged contact between the macrophages and T-cells, since activation is optimal after a 4-hr incubation. In addition, our results clearly show that the T-cell activation process, which is antigen-dependent, is separable from the production of helper function, which occurs in the absence of additional antigen.

*Summary.* In this report we investigated the ability of macrophage-associated OVA to stimulate purified OVA-immune T-lymphocytes to produce nonspecific helper activity for antibody production. T-cell activation required only a brief contact between the antigen-pulsed macrophages and T-lymphocytes and was maximal after a 4-hr contact. T-cell activation occurred at 37 but not at 4°C, indicating that the macrophages and/or lymphocytes must be metabolically active for efficient activation. In addition, we compared the ability of antigen-pulsed syngeneic or allogeneic macrophages to activate T-cells for helper function.

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