Proc. Natl. Acad. Sci. USA
Vol. 74, No. 5, pp. 2104–2108, May 1977
Immunology

Nature of the antigenic complex recognized by T lymphocytes: Specific sensitization by antigens associated with allogeneic macrophages*

(major histocompatibility complex/mixed leukocyte reaction)

DAVID W. THOMAS AND ETHAN M. SHEVACH

Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

Communicated by David W. Talmage, March 4, 1977

ABSTRACT **Alloreactive guinea pig thymus-derived (T) cells generated *in vitro* were rendered unresponsive to allogeneic macrophages by treatment with bromodeoxyuridine and light. The remaining T cells were subsequently primed and rechallenged in tissue culture with trinitrophenyl (Tnp)-modified syngeneic or allogeneic macrophages. By this procedure we found that the remaining T cells primed with Tnp-modified allogeneic macrophages could be restimulated only with Tnp-modified allogeneic, not syngeneic, macrophages. Similarly, if the remaining T cells were primed with Tnp-modified syngeneic macrophages, they could be restimulated only by Tnp-modified syngeneic, and not by allogeneic, macrophages. In contrast, no T cell sensitization with Tnp-modified syngeneic or allogeneic macrophages occurred if the alloreactive T cells were treated with light alone, suggesting that an uninhibited mixed leukocyte reaction causes nonspecific suppression of antigen-specific T cell priming. These results indicate that the genetic restriction of T cell–macrophage interactions is imposed by the type of macrophage used for initial sensitization rather than by a requirement for self-recognition through cellular interaction structures.**

Two concepts have been proposed to explain the histocompatibility restrictions of immunocompetent cell interactions of thymus-derived (T) lymphocytes with both macrophages (1) and bone-marrow-derived (B) lymphocytes (2). According to one proposal (the cellular interaction structure model), major histocompatibility (MHC) genes code for specific cellular interaction structures and homology between these structures is necessary for effective cell interactions. As suggested by Shevach (3), these cellular interaction structures in the guinea pig may be antigens associated with the I region (Ia). According to this model, T cells would exhibit dual recognition by virtue of binding to antigen-pulsed macrophages through their antigen-specific receptors and their cellular interaction structures. A second proposal (the complex antigenic determinant model) is based on observations that mouse T cells sensitized to hapten- or virus-modified cells are primarily cytotoxic for similarly modified target cells that are H-2K or H-2D compatible (4, 5). These observations have been interpreted to indicate that T cells do not recognize antigens *per se*, but can only be sensitized to antigen-modified membrane components or to complexes of antigen combined with certain membrane molecules. One prediction of this latter hypothesis is that MHC homology is not necessary for effective T cell–macrophage interactions, but that T cells recognize antigens associated only with the macrophage histocompatability type used for initial sensitization.

In a previous report, we showed that $(2 \times 13)F_1$ guinea pig T cells which had been initially primed *in vitro* with antigen-pulsed macrophages derived from one parent could be restimulated in a second culture only with antigen-pulsed macrophages derived from the parent used for initial sensitization, and not with antigen-pulsed macrophages from the other parent (6). This result suggests that the primary restriction on the F_1 T cell response may be imposed by the type of macrophage used for initial sensitization, irrespective of the Ia antigen which that macrophage expressed. Nevertheless, the cellular interaction structure proposal could not be ruled out by these studies because the F_1 T cell and parental macrophages share Ia specificities. Although we previously attempted to demonstrate T cell sensitization to antigen-pulsed allogeneic macrophages, the results were difficult to interpret due to the magnitude of the mixed leukocyte reaction (MLR). For this reason we developed a technique in which the MLR could be eliminated by bromodeoxyuridine and light treatment prior to priming the T cells with antigens associated with allogeneic macrophages. By this procedure we found that T cells primed *in vitro* with antigen-treated allogeneic macrophages can be restimulated only with antigen associated with allogeneic, and not with syngeneic macrophages, and that antigen-specific sensitization can be obtained only in the absence of an MLR.

MATERIALS AND METHODS

Animals. Inbred strain 2 and strain 13 guinea pigs were obtained from the Division of Research Services, National Institutes of Health, Bethesda, MD.

Preparation of Cells. Guinea pigs were injected intraperitoneally with 25 ml of sterile mineral oil (Marcol 52, Humble Oil and Refining Co., Houston, TX) and the resulting peritoneal exudate was harvested 3–4 days later. This cell population, consisting of approximately 75% macrophages, 10% neutrophils, and 15% lymphocytes, was used as a source of macrophages for antigen pulsing (see below). A T-lymphocyte-enriched cell population was prepared by passing lymph node cells, from animals injected in the footpads several weeks previously with complete Freund's adjuvant (Difco Laboratories, Detroit, MI), over a rayon wool adherence column (7).

Bromodeoxyuridine and Light Treatment (8). Strain 13 T cells (6×10^6) were cultured with strain 2 macrophages (2×10^6) in 3 ml final volume of RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY) containing L-glutamine (300 μg/ml), penicillin (100 units/ml), 5-fluorocytosine (5 μg/ml), 2-mercaptoethanol (50 μM), and 5% heat-inactivated normal guinea pig serum at 37° in 5% CO_2 in air. 5-Bromo-2′-deoxyuridine (BrdUrd, 2 μg/ml, Nutritional Biochemical Corp.,

Abbreviations: T lymphocytes, thymus-derived lymphocytes; B lymphocytes, bone-marrow-derived lymphocytes; BrdUrd, 5-bromo-2′-deoxyuridine; [^{3}H]dThd, tritiated thymidine; Ia, I-region-associated antigens; MHC, major histocompatibility complex; MLR, mixed leukocyte reaction; Tnp, 2,4,6-trinitrophenyl.

* This is paper III in a series. The preceding paper is: Thomas, D. W. & Shevach, E. M. (1977) "Nature of the antigenic complex recognized by T lymphocytes. II. T cell activation by direct modification of macrophage histocompatibility antigens," *J. Exp. Med.*, in press.

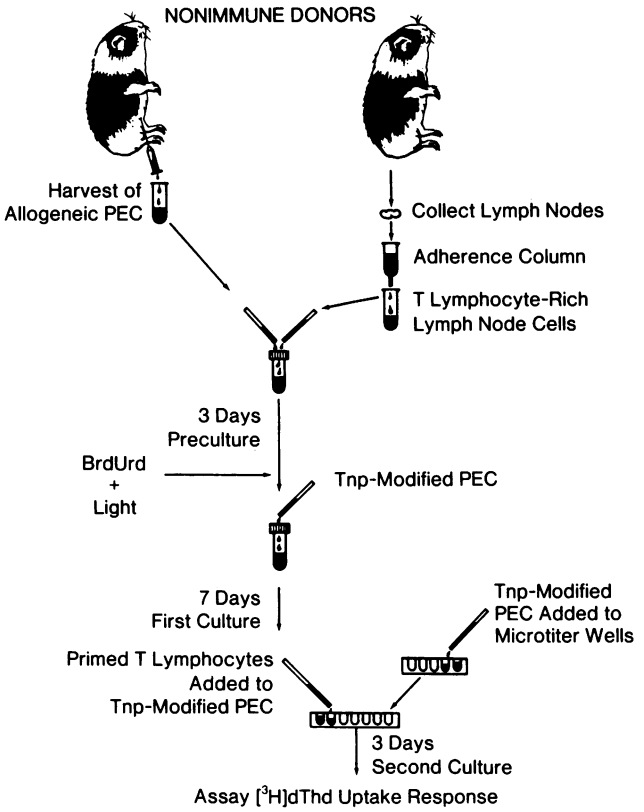

FIG. 1. Schematic diagram for the bromodeoxyuridine (BrdUrd) and light treatment of alloreactive guinea pig T lymphocytes and subsequent *in vitro* priming with antigen-pulsed macrophages. The details of this procedure are described under *Materials and Methods*. PEC, peritoneal exudate cells.

Cleveland, OH) was added to the cultures at 48 hr, and at 72 hr the cultures were illuminated 90 min by a fluorescent light source to eliminate the alloreactive T cells stimulated to synthesize DNA during the 3 day "preculture" (Fig. 1). This procedure diminished the response of the strain 13 T cells to strain 2 macrophages by approximately 80%.

***In Vitro* Antigen Priming.** Unfractionated peritoneal exudate cells (5 to 10 × 10^6/ml) were incubated for 1 hr at 37° in Hanks' balanced salt solution containing 25 μg/ml of mitomycin C (Sigma Chemical Co., St. Louis, MO), then washed four times to remove the unbound mitomycin. The mitomycin-C-treated macrophages were treated with 2,4,6-trinitrobenzene sulfonate according to Shearer *et al.* (5) and 1 × 10^6 of the trinitrophenyl (Tnp)-modified macrophages added to BrdUrd- and light-treated T cells for primary sensitization (Fig. 1). The primary cultures were maintained in a total volume of 1.5 ml RPMI + 5% normal guinea pig serum. During the first culture the cells were incubated for 1 week at 37° in 5% CO_2 in air and on the third day of culture the medium was decanted and replaced with 1.5 ml of fresh medium.

***In Vitro* Assay of DNA Synthesis.** The antigen-primed T cells (1 to 2 × 10^5 per well) recovered from the first culture were restimulated in a second culture in round-bottom microtiter plates (Cooke Engineering, Arlington, VA) with fresh Tnp-modified peritoneal exudate cells (1 × 10^5 per well) in a total volume of 0.2 ml of RPMI medium containing 5% normal guinea pig serum. After incubation for 2 days at 37° in 5% CO_2 in air, 1 μCi of tritiated thymidine ([^{3}H]dThd, specific activity 6.7 Ci/mmol, New England Nuclear Corp., Boston, MA) was added to each well (Fig. 1). The amount of radioactivity incorporated into cellular DNA was determined after an additional 18 hr incubation with the aid of a semiautomated microharvesting device. The results of triplicate cultures are expressed as total cpm per culture.

RESULTS AND DISCUSSION

BrdUrd and light treatment of alloreactive strain 13 T cells generated during a 3 day preculture with strain 2 macrophages reduced the subsequent MLR response against strain 2 macrophages approximately 80%, when compared to cells treated with light alone (Table 1). The BrdUrd treatment was not generally toxic, because the remaining strain 13 T cells were capable of being sensitized with Tnp-modified syngeneic or allogeneic macrophages. Thus, strain 13 T cells precultured with strain 2 macrophages and treated with BrdUrd and light were capable of being primed in the first culture with Tnp-modified strain 13 macrophages and showed a response to Tnp-modified strain 13, but not strain 2, macrophages upon restimulation in the second culture (Table 1, experiments 3–5). This priming was not dependent on a helper effect produced by preculture with allogeneic strain 2 macrophages, because similar results were obtained with strain 13 T cells precultured with syngeneic macrophages (Table 1, experiments 1 and 2). In addition, BrdUrd- and light-treated strain 13 T cells rendered unresponsive to strain 2 alloantigens could also be specifically primed with Tnp-modified allogeneic strain 2 macrophages and upon restimulation showed a good response to Tnp-modified strain 2 macrophages, while showing little or no response with Tnp-modified syngeneic strain 13 macrophages. In contrast, no Tnp-specific stimulation was observed with strain 13 T cells treated with light alone and primed with Tnp-modified allogeneic macrophages. In this case a very substantial secondary MLR occurred. Attempts to eliminate the MLR of strain 2 T cells stimulated with strain 13 macrophages with BrdUrd and light were not successful due to the delayed kinetics and reduced magnitude of the strain 2 against 13 MLR (6, 9). However, under conditions where the strain 2 against 13 MLR has been reduced by anti-Ia sera against the strain 13 macrophages we could directly demonstrate that strain 2 T cells can be specifically sensitized to antigens associated with the strain 13 macrophages (see paper II in this series*).

A summary of the results demonstrating specific T cell sensitization with Tnp-modified allogeneic macrophages (from Table 1) is shown in Table 2. BrdUrd- and light-treated strain 13 T cells primed with Tnp-modified allogeneic strain 2 macrophages showed an average of 46,450 Δcpm upon restimulation with Tnp-modified strain 2 macrophages, and only 4800 Δcpm when restimulated with Tnp-modified strain 13 macrophages. In similar fashion, strain 13 T cells initially sensitized with Tnp-modified syngeneic strain 13 macrophages showed 27,950 Δcpm upon restimulation with Tnp-modified strain 13 macrophages, but only 5590 Δcpm when restimulated with Tnp-modified strain 2 macrophages. The T cell preference for the Tnp-modified macrophage used for initial sensitization is likewise reflected in the average stimulation indices. Thus, the stimulation index to Tnp-modified strain 2 macrophages was 2.8 ± 0.3 with T cells primed with Tnp-modified strain 2 macrophages but only 1.1 ± 0.1 with T cells primed with Tnp-modified strain 13 macrophages. Likewise, the stimulation index to Tnp-modified strain 13 macrophages was 7.5 ± 2.8 with T cells primed with Tnp-modified strain 13 macrophages and 1.6 ± 0.3 with T cells primed with Tnp-modified strain 2 macrophages. The reason the stimulation index of strain 13 T cells primed and restimulated with Tnp-modified strain 2 macrophages is somewhat lower than that of strain 13 T cells primed and restimulated with Tnp-modified strain 13 T cells

Table 1. Specific T lymphocyte sensitization by Tnp-modified allogeneic macrophages

Exp.	Preculture* T lymphocyte	Preculture* Macrophage	Preculture* BrdUrd + light	First culture† Primed with macrophage strain	Second culture,‡ [^{3}H]dThd cpm for macrophage strain 2 Untreated	2 Tnp	13 Untreated	13 Tnp
1	13	13	−	13-Tnp	16,930	24,260	9,600	*78,600*
	13	2	−	2-Tnp	395,800	271,200	97,400	99,500
	13	2	+	2-Tnp	68,590	*161,280*	13,810	31,670
2	13	13	−	13-Tnp	1,470	770	1,930	*6,010*
	13	2	−	2-Tnp	46,530	51,520	7,630	7,510
	13	2	+	2-Tnp	7,610	*24,020*	4,110	3,900
3	13	2	−	2-Tnp	233,840	216,570	99,470	80,890
	13	2	+	2-Tnp	20,820	*77,200*	13,980	12,820
	13	2	+	13-Tnp	34,100	47,160	9,640	*37,010*
4	13	2	+	2-Tnp	56,930	*112,240*	4,240	11,930
	13	2	+	13-Tnp	68,520	75,030	1,230	*22,300*
5	13	2	−	2-Tnp	17,150	20,170	4,770	5,180
	13	2	+	2-Tnp	4,500	*11,020*	4,810	6,490
	13	2	+	13-Tnp	4,800	5,840	5,760	*24,010*
6	13	2	+	2-Tnp	23,450	*75,420*	6,030	7,590

*Strain 13 T cells were cultured for 3 days with strain 2 or strain 13 macrophages and treated with bromodeoxyuridine (BrdUrd) and light (+) or light alone (−), as described under *Materials and Methods.*

† The cells remaining from the preculture were primed by culture for 7 days with Tnp-modified strain 2 or strain 13 macrophages as described under *Materials and Methods.*

‡ Primed T cells recovered from the first culture were restimulated in the second culture with untreated or Tnp-modified strain 2 or strain 13 macrophages and the incorporation of [^{3}H]dThd was determined after an additional 3 day culture as described under *Materials and Methods.* Italicized values indicate cultures in which a positive response has occurred.

(2.8 ± 0.3 versus 7.5 ± 2.8) is that the background cpm with untreated allogeneic strain 2 macrophages is elevated due to the residual MLR, which is unrelated to Tnp-specific stimulation. For this reason we consider the Δcpm to more accurately reflect Tnp-specific stimulation and by this criteria strain 13 T cells primed and restimulated with Tnp-modified allogeneic strain 2 macrophages show about the same response as strain 13 T cells primed and rechallenged with Tnp-modified syngeneic strain 13 macrophages (46,450 versus 27,950 Δcpm).

The failure to obtain sensitization to Tnp-modified allogeneic macrophages in the presence of an MLR may reflect a direct suppression of antigen-specific priming resulting from the allogeneic response. Thus, we found that if strain 13 T cells were cultured with strain 2 macrophages, they failed to become sensitized upon culture with Tnp-modified strain 13 macrophages (Table 3). In contrast, strain 13 T cells precultured with strain 2 macrophages and treated with BrdUrd and light to render them unresponsive to strain 2 alloantigens were capable of being sensitized with Tnp-modified strain 13 macrophages and showed a good response upon restimulation with Tnp-modified strain 13 macrophages in the second culture (Table 3). This apparent nonspecific suppression occurring during an MLR may explain our previous failure to detect specific T cell sensitization by antigens associated with allogeneic macrophages when the MLR was not inhibited. Several other investigators (10, 11) have reported a similar nonspecific suppressor effect by alloreactive mouse T cells for antigen-specific or MLR reactions and have shown that this suppression is mediated by a soluble inhibitory factor. It is therefore possible that the MLR-induced suppression of guinea pig T cell priming we observed is produced by a similar mechanism; it should be enlightening to determine if alloreactive guinea pig T cells release a soluble suppressor.

Table 2. Summary of results demonstrating T lymphocyte sensitization with Tnp-modified allogeneic macrophages

Strain 13 T cells primed with Tnp-modified macrophage strain	Response, Δcpm* for Tnp-modified macrophage strain 13-Tnp	2-Tnp
13-Tnp	*27,950* ± 10,950 (7.5 ± 2.8)†	5,590 ± 2,360 (1.1 ± 0.2)†
2-Tnp	4,800 ± 2,860 (1.6 ± 0.3)†	*46,450* ± 12,630 (2.8 ± 0.3)†

*The data for this table were obtained from Table 1 and represent the mean of five (priming with Tnp-13 macrophages) or six (priming with Tnp-2 macrophages) experiments ± the standard error. The Δcpm was calculated by subtracting the cpm from cultures with untreated macrophages from cultures stimulated with Tnp-modified macrophages.

† Values in parentheses represent the mean of the stimulation index ± the standard error. The stimulation index was calculated by dividing the cpm from cultures stimulated with Tnp-modified macrophages by the cpm obtained from cultures with untreated macrophages.

Because strain 2 and strain 13 guinea pigs differ only in the I region of the MHC, our results strongly suggest that Ia homology is not required for efficient T cell–macrophage collaboration in response to antigen. Rather, the genetic restriction of this interaction may be imposed only by the histocompatibility type of the macrophage used for initial T cell sensitization. In this regard, these results would support the complex antigenic determinant concept of T cell recognition. Several investigators have reached a similar conclusion for immunocompetent cell interactions in the mouse (12–14). An

Table 3. Inhibition of T lymphocyte sensitization by the mixed leukocyte reaction

Exp.	Preculture* T lymphocyte	Preculture* Macrophage	Preculture* BrdUrd + light	First culture† Primed with macrophage strain	Second culture,‡ [^{3}H]dThd cpm for macrophage strain: 2 Untreated	2 Tnp	13 Untreated	13 Tnp
1	13	2	−	13-Tnp	303,200 ±22,430	281,100 ±15,780	14,850 ±1,560	28,160 ±2,930
	13	2	+	13-Tnp	133,170 ±10,420	151,270 ±8,360	28,380 ±3,200	*276,900* ±9,320
2	13	2	−	13-Tnp	209,710 ±32,760	235,700 ±19,063	14,070 ±1,033	20,100 ±1,825
	13	2	+	13-Tnp	34,100 ±6,170	47,160 ±1,074	9,640 ±240	*37,010* ±477

* Strain 13 T cells were cultured for 3 days with strain 2 macrophages and treated with BrdUrd and light (+) or light alone (−), as described under *Materials and Methods.*

† The cells remaining from the preculture were primed by culture for 7 days with Tnp-modified strain 13 macrophages as described under *Materials and Methods.*

‡ Primed T cells recovered from the first culture were restimulated in the second culture with untreated or Tnp-modified strain 2 or strain 13 macrophages and the incorporation of [^{3}H]dThd was determined after an additional 3 day culture as described under *Materials and Methods* (mean cpm ± the standard error). Italicized values indicate cultures in which a positive response has occurred.

alternative explanation (15) for the successful interactions between histoincompatible immunocompetent cells derived from tetraparental or radiation chimeric mice (12, 16) is that immature lymphoid cells undergo adaptive changes during their development *in vivo* that allow successful collaboration of the mature cells. Thus, in response to antigen the mature lymphocytes would collaborate only with cell types present during their development, but not with cells with which they have had no contact during ontogeny. Our finding that mature T cells may become specifically sensitized to antigen associated with allogeneic macrophages *in vitro* rules against this possibility, unless such an adaptive process can occur with mature T cells under short-term culture conditions.

One implication of the complex antigenic determinant theory is that for antigens under *Ir* gene control it might be expected that T cells would be capable of being sensitized with the genetically controlled antigens associated with macrophages of the responder or nonresponder haplotype. However, Shevach and Rosenthal (1) and Shevach (3) found that in systems controlled by *I*-linked immune response genes, immune (nonresponder × responder)F_1 T cells could not be activated by antigens associated with macrophages of the nonresponder parent. One explanation for this finding is that the Ia antigens are the *Ir* gene products and function as antigen recognition structures on macrophages. Thus, nonresponder macrophages lack the *I*-region gene products necessary to process and/or present the corresponding genetically controlled antigen. To examine this possibility, it should be possible to sensitize nonresponder T cells with antigen-pulsed macrophages derived from responder animals expressing the corresponding antigen-specific *I*-region gene products. If specific sensitization occurs under these conditions it would support the proposal of *Ir* gene expression by macrophages.

An alternate explanation for the results presented in this study may be that while Ia antigen-mediated self-recognition is not required for efficient collaboration, T cells may still exhibit dual recognition by virtue of expression of both a recognition structure for self or foreign Ia antigens (an anti-Ia receptor) and an antigen-specific receptor. According to this proposal, T cells may bind to macrophage Ia antigens through their anti-Ia receptor and to macrophage-bound antigen through their antigen-specific receptor. Both receptors would have to be bound to activate the T cell. In order for this theory to explain the failure of nonresponder macrophages to activate immune (nonresponder × responder)F_1 T cells, one must postulate that the anti-Ia receptor of F_1 T cells must be expressed as a functional unit with the antigen-specific receptor. That is, in F_1 T cells the anti-Ia receptor capable of recognizing the nonresponder haplotype would never be associated with the specific receptor for antigen, the response to which is controlled by the responder haplotype. One possibility is that this association occurs as a result of physical linkage between the anti-Ia and antigen-specific receptors on the F_1 T cell. Alternatively, F_1 T cells might exhibit allelic exclusion for the expression of anti-Ia receptors. That is, in an F_1 animal a distinct population of T cells expressing the antigen-specific receptor of the responder haplotype would express anti-Ia receptors directed only against the responder haplotype, but not against the nonresponder haplotype.

The authors are grateful to Mr. Charles Hoes for his expert technical assistance and to Dr. W. E. Paul for many helpful discussions. D.W.T. was supported by Fellowship F32 AI 05141-01 from the National Institute of Allergy and Infectious Diseases.

The costs of publication of this article were defrayed in part by the payment of page charges from funds made available to support the research which is the subject of the article. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

1. Shevach, E. M. & Rosenthal, A. S. (1973) *J. Exp. Med.* **138** 1213–1229.
2. Katz, D. H. & Benacerraf, B. (1975) *Transplant. Rev.* **22** 175–203.
3. Shevach, E. M. (1976) *J. Immunol.* **116,** 1482–1489.
4. Zinkernagel, R. M. & Doherty, P. C. (1975) *J. Exp. Med.* **141,** 1427–1436.
5. Shearer, G. M., Rehn, T. G. & Garbarino, C. A. (1975) *J. Exp. Med.* **141,** 1348–1364.
6. Thomas, D. W. & Shevach, E. M. (1976) *J. Exp. Med.* **144,** 1263–1273.

7. Rosenstreich, D. L., Blake, J. T. & Rosenthal, A. S. (1971) *J. Exp. Med.* **134,** 1170–1186.
8. Janeway, C. A., Jr. & Paul, W. E. (1976) *J. Exp. Med.* **144,** 1641–1656.
9. Greineder, D. K., Shevach, E. M. & Rosenthal, A. S. (1976) *J. Immunol.* **117,** 1261–1274.
10. Rich, S. S. & Rich, R. R. (1975) *J. Exp. Med.* **142,** 1391–1402.
11. Gisler, R. H. & Fridman, W. H. (1975) *J. Exp. Med.* **142,** 507–511.
12. Pfizenmaier, K., Starzinski-Powitz, A., Root, H. N., Röllinghoff, M. & Wagner, H. (1976) *J. Exp. Med.* **143,** 999–1003.
13. Pierce, C. W., Kapp, J. M. & Benacerraf, B. (1976) *J. Exp. Med.* **144,** 371–381.
14. Miller, J. F. A. P., Vadas, M. A., Whitelaw, A. & Gamble, J. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2486–2490.
15. Katz, D. H. (1976) *J. Immunol.* **117,** 1853–1859.
16. Bechtol, K. B., Freed, J. H., Herzenberg, L. A. & McDevitt, H. O. (1974) *J. Exp. Med.* **140,** 1660–1675.