Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 6628–6631, November 1983
Immunology

Activation of antigen-specific B cells: Role of T cells, cytokines, and antigen in induction of growth and differentiation

(thymus-dependent antigens/linked recognition)

RANDOLPH J. NOELLE, E. CHARLES SNOW, JONATHAN W. UHR, AND ELLEN S. VITETTA

Department of Microbiology, University of Texas Health Science Center, Dallas, Texas 75235

Communicated by David W. Talmage, June 22, 1983

ABSTRACT **T cells and cytokines were used to activate highly enriched populations of 2,4,6-trinitrophenyl (TNP)-binding B cells (TNP-ABC). TNP-ABC did not proliferate or differentiate when they were cultured with thymus-dependent (TD) antigen, even in the presence of supernatants known to contain B-cell growth and differentiation factors. However, purified TNP-ABC did proliferate and differentiate when they were cultured with TD antigen in the presence of carrier-primed T cells and antigen (TNP-keyhole limpet hemocyanin)—i.e., linked recognition. TNP-ABC blasts generated under conditions of linked recognition proliferated and differentiated in response to cytokines in the absence of T cells and antigen. In contrast, under conditions of nonlinked recognition (hapten and carrier on different molecules) TNP-ABC blasts also proliferated but did not differentiate in response to the same cytokines. These results indicate that antigen-specific "resting" B cells must be activated by T cells and antigen prior to becoming responsive to cytokines. Furthermore, activation under conditions of linked and nonlinked recognition generates two different types of blasts with regard to their subsequent response to cytokines.**

The induction of maximal B-cell plaque-forming cell (PFC) responses to certain soluble antigens requires the participation of H-2-restricted antigen-specific T helper cells [hence the antigens are called "thymus-dependent" (TD)] and the appropriate hapten-carrier conjugates (linked recognition) (1–5). The role of cytokines in inducing or sustaining the proliferation and differentiation of B cells responding to TD antigens has not been extensively studied. In general, supernatants containing T cell-derived lymphokines that act on B cells have been described in polyclonal systems (6–18) or in systems using erythrocyte-bound antigens (7, 8).

In this study we have utilized a population of purified 2,4,6-trinitrophenyl (TNP)-antigen-binding B cells (TNP-ABC) in a TD antigen-driven system to analyze the effect(s) of T cells and cytokines. The major advantage of this system, compared to the use of non-antigen-enriched B-cell populations, is that B-cell growth as well as differentiation can be assessed. In addition, because the B cells are >80% TNP specific, responding TNP-specific B-cell blasts can be visualized and isolated. As described previously (19), when T cells primed to keyhole limpet hemocyanin (KLH) are cultured with antigen (TNP-KLH) and TNP-binding B cells from unprimed mice, B-cell proliferation and differentiation occur.

The data presented in this communication indicate that cytokines (with or without antigen) do not induce the differentiation or proliferation of "resting" TNP-ABC. However, when the TNP-ABC are first activated by means of linked recognition, the resultant blasts both proliferate and differentiate in response to cytokines; when activation occurs by means of nonlinked recognition, the blasts proliferate but do not differentiate in response to the same cytokines.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Animals. Male or female BALB/c mice (Cumberland Farms, Clinton, TN), 8–12 weeks of age, were used for all experiments.

Preparation of Cells. The methods used for the isolation of TNP-specific B cells have been described (20). T cells were obtained from the popliteal lymph nodes of mice immunized 5–8 days previously in the hind footpads with 50–75 μg of KLH (Calbiochem–Behring). Between 90% and 95% of the B cells were removed by adherence to Petri dishes coated with affinity-purified rabbit anti-mouse Ig (RAMIg) (21). Immediately prior to use, the T cells were irradiated (1,500 rads; 1 rad = 0.01 gray).

Culture Conditions. Culture conditions have been described (19).

Generation of Activated TNP-ABC (TNP-ABC*). TNP-ABC were cultured with primed T cells ($3–4 \times 10^5$ cells per well, in 0.2 ml) and antigen [TNP-KLH or fluoresceinated KLH (Flu-KLH) and TNP-human serum albumin (TNP-HSA)] for 42–48 hr. Cells in individual microwells were harvested and pooled. The dead cells and soluble antigen were removed by centrifugation through 30% Percoll (Pharmacia). To remove irradiated T cells that persisted in culture after 48 hr, the cells were washed twice with 5% fetal calf serum in a balanced salt solution (7) and were treated with monoclonal anti-Thy 1.2 antibody (H013.12) (10) for 45 min at 4°C. Cells were washed and incubated with absorbed baby rabbit complement for 20 min at 37°C. The remaining viable cells were isolated by flotation on Ficoll/Hypaque (Pharmacia) and cultured at 25,000 cells per well.

Preparation of Cytokines. EL-4 thymoma cells were cultured with phorbol 12-myristate 13-acetate (PMA) as described (22). The cell-free supernatant was brought to 80% saturation with $(NH_4)_2SO_4$. The pellet was dissolved in phosphate-buffered saline to 10% of the original volume, sterilized by filtration, and stored at −20°C. Human interleukin 1 (IL-1) was obtained from Genzyme (Norwalk, CT) and used at 5 units/ml.

Analysis of Cell Size. Cell size was estimated by forward low-angle light scatter on an Ortho Diagnostics Cytofluorograf flow cytometer. Each histogram was generated by the analysis of at least 10,000 cells.

RESULTS

Effect of Primed T Cells and Antigen on the Proliferation and Differentiation of TNP-ABC. As previously reported (19) and as confirmed in this study (Fig. 1 *Left*), on day 3 of culture, maximal proliferation of TNP-ABC was induced in the presence of irradiated, purified KLH-primed T cells and TNP-KLH

Abbreviations: PFC, plaque-forming cell; TD, thymus-dependent; TNP, 2,4,6-trinitrophenyl; TNP-ABC, TNP-antigen-binding cells; TNP-ABC*, activated TNP-ABC; TNP-HRBC, TNP-horse erythrocytes; TNP-HSA, TNP-human serum albumin; KLH, keyhole limpet hemocyanin; Flu-KLH, fluoresceinated KLH; RAMIg, rabbit anti-mouse immunoglobulin; PMA, phorbol 12-myristate 13-acetate; IL-1, interleukin-1.

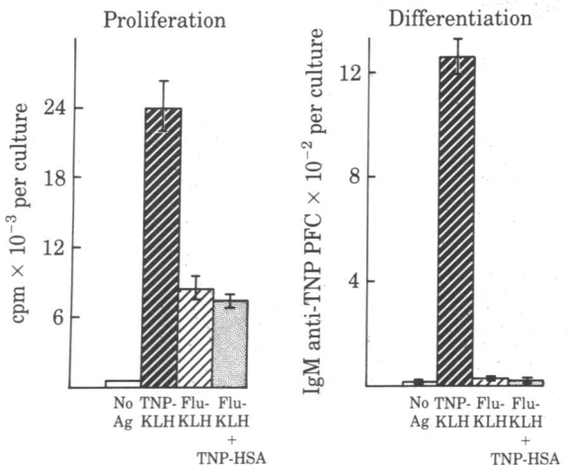

FIG. 1. Induction of B-cell differentiation and proliferation by primed T cells and antigen. Irradiated primed T cells (4×10^5 cells per culture) were cultured with TNP-ABC (5×10^4 cells per culture) in the presence of TNP-KLH (200 ng/ml), Flu-KLH (200 ng/ml) plus TNP-HSA (200 ng/ml), or no antigen (Ag). Cultures were either pulsed with 1 μCi (3.7×10^4 Bq) of [^{3}H]thymidine for 16 hr on day 3 and assessed for proliferative activity (*Left*) or assayed for IgM anti-TNP PFC on day 5 (*Right*). The data are expressed as the mean ± SD of triplicate cultures.

(linked recognition). In the presence of Flu-KLH, TNP-HSA, or both and KLH-primed T cells (nonlinked recognition), B-cell proliferation was also induced; it accounted for approximately 30% of the response obtained with linked recognition. When TNP-ABC were cultured with purified KLH-primed T cells under conditions of linked recognition, direct anti-TNP PFC were generated on day 5 (Fig. 1, *Right*); activation by nonlinked recognition was ineffective at inducing PFC. In the absence of antigen, primed T cells did not induce anti-TNP PFC. Therefore, with regard to T-cell help, proliferation but not differentiation of B cells can be induced by nonlinked recognition.

Effect of Cytokines on the Growth and Differentiation of TNP-ABC. Because T cells are effective at inducing both the replication and differentiation of TNP-ABC under conditions of linked recognition, it was important to determine whether T-cell-derived lymphokines or IL-1 could be substituted for T cells. The source of T-cell-derived lymphokines was the supernatant of PMA-activated EL-4 cells (22). The addition of up to 10% EL-4 supernatant or IL-1 had no antigen-dependent stimulatory effect on the growth or differentiation of TNP-ABC (Table 1). The modest effect of the cytokines alone on growth and differentiation can probably be attributed to the small number of B cells activated *in vivo* that are present in the spleen cell suspensions of these mice. Thus, cytokines could not replace carrier-primed T cells in their ability to induce antigen-specific proliferation and differentiation of the TNP-ABC.

Table 1. Effects of cytokines on the proliferation and differentiation of TNP-ABC

Addition to culture*	[^{3}H]Thymidine incorporation,† cpm per culture		IgM anti-TNP,‡ PFC per culture	
	Control	With TNP-KLH§	Control	With TNP-KLH§
None	823 ± 47	702 ± 83	0	0
EL-4 SN	2,250 ± 125	1,641 ± 109	0	20 ± 14
IL-1	933 ± 64	913 ± 24	0	0
EL-4 SN + IL-1	2,544 ± 254	3,246 ± 381	5 ± 4	8 ± 6
Carrier-primed T cells	708 ± 56	11,605 ± 397	2 ± 0	1,312 ± 12

* EL-4 SN was 2 μl per culture; IL-1 was 1 unit per culture; primed T cells were 4×10^5 per culture.
† Cultures were pulsed with 1 μCi of [^{3}H]thymidine 36–48 hr after the initiation of culture. The results are expressed as the arithmetic mean ± SD of triplicate cultures.
‡ Cultures were assessed for direct anti-TNP-PFC on day 5 of culture. The results are expressed as the arithmetic mean ± SD of triplicate cultures.
§ Two hundred nanograms of TNP-KLH was added.

After Activation by Linked Recognition, TNP-ABC Proliferate and Differentiate in Response to Cytokines. Because EL-4 supernatant and IL-1 were ineffective at *initiating* an antigen-driven B-cell response, the cytokines were tested for their capacity to *sustain* a response initiated by T cells plus antigen. TNP-ABC were cultured for 48 hr with irradiated primed T cells and TNP-KLH. The cultures were harvested and the persisting irradiated T cells (5% of input cells) were eliminated with monoclonal anti-Thy-1 antibody and complement. The remaining viable cells were isolated and used as the source of TNP-ABC*. The TNP-ABC* were >98% surface immunoglobulin-positive and consisted of a heterogeneous population of B cells that were predominantly large lymphocytes (Fig. 2 *Lower*). In contrast, the nonactivated TNP-ABC were a relatively homogeneous population of small lymphocytes (Fig. 2 *Upper*). Each cell population was cultured at 25,000 cells per well and tested for cytokine responsiveness.

The TNP-ABC and the TNP-ABC* blasts activated by linked recognition were compared for their ability to respond to cy-

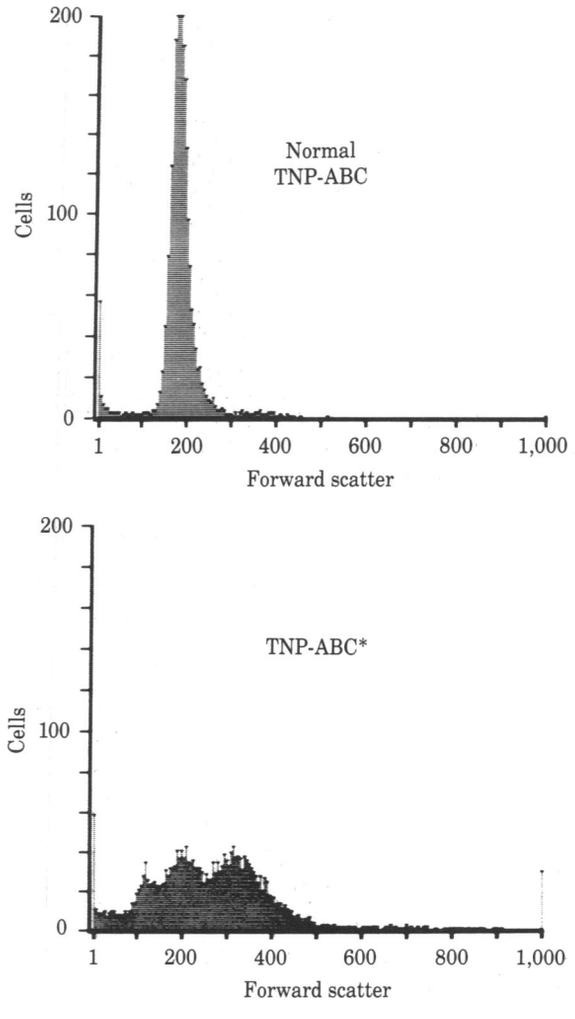

FIG. 2. Cell size analysis of normal and activated TNP-ABC. Either normal TNP-ABC (*Upper*) or TNP-ABC* (*Lower*) were analyzed for forward light scatter. Scatter was analyzed with 10,000 cells per group.

tokines in the absence of antigen and T cells. Growth was assessed by the incorporation of [^{3}H]thymidine and by cell recovery. Differentiation was determined by direct anti-TNP PFC on day 6 (4 days after the *in vitro* activation of the TNP-ABC). As seen in Fig. 3, TNP-ABC neither replicated nor differentiated when EL-4 supernatant, IL-1, or both were added to the cultures. In contrast, the TNP-ABC* were responsive to both EL-4 supernatant and IL-1. Thus, the addition of EL-4 supernatant to the cultures of TNP-ABC* blasts increased the incorporation of [^{3}H]thymidine 2.4-fold and the recovery of cells 4.8-fold. EL-4 supernatant enhanced the anti-TNP-PFC response of the TNP-ABC* blasts 16-fold. IL-1 alone also exhibited similar effects—i.e., 2.5- and 3.4-fold enhancement of [^{3}H]thymidine incorporation and cell recovery, respectively, as well as a 10-fold increase in anti-TNP PFC. The effects of EL-4 and IL-1 were approximately additive—i.e., a 3.9- and 9-fold increase of [^{3}H]thymidine incorporation and cell recovery, respectively, and a 31-fold increase in PFC generation. The results indicate that, once the TNP-ABC are activated by linked recognition, they are rendered responsive to cytokines.

After Activation by Nonlinked Recognition, TNP-ABC Proliferate but Do Not Differentiate in Response to Cytokines. Fig. 1 illustrated that after the addition of carrier or carrier plus hapten (Flu-KLH plus TNP-HSA), to the TNP-ABC, proliferation occurred in the virtual absence of PFC generation. To determine whether the growth of TNP-ABC* activated by nonlinked recognition was cytokine mediated, the TNP-ABC* blasts were isolated and tested for their capacity to proliferate and differentiate in response to EL-4 supernatant and IL-1. These blasts proliferated but did not differentiate in response to the cytokines (Fig. 4). Therefore, TNP-ABC* blasts activated under conditions of nonlinked recognition respond differently to cytokines than do TNP-ABC* blasts activated under conditions of linked recognition.

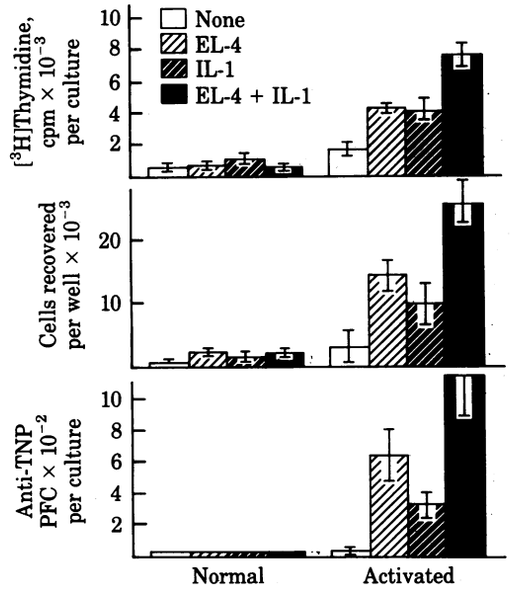

FIG. 3. Cytokine-induced growth and differentiation of TNP-ABC* blasts activated by linked recognition. TNP-ABC or TNP-ABC* blasts activated by linked recognition were incubated at 25,000 cells per culture. To each culture, EL-4 SN (1% vol/vol), IL-1 (5 units/ml), EL-4 supernatant plus IL-1, or no cytokines were added. After an additional 4 days of culture, cells were assessed for growth by counting viable cells and by pulsing each culture with 1 μCi of [^{3}H]thymidine for 16 hr. Differentiation was assessed by determining IgM anti-TNP PFC on aliquots of the same cultures.

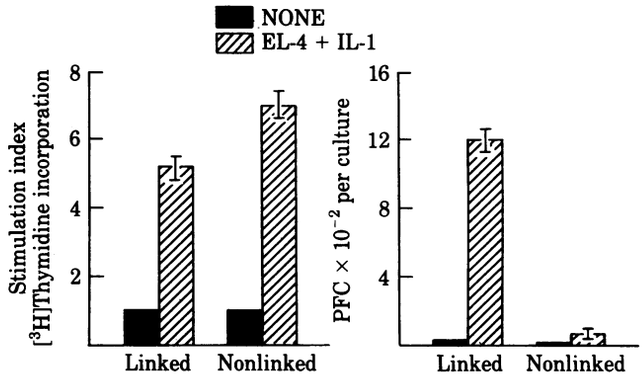

FIG. 4. Cytokine-induced growth and differentiation of TNP-ABC* blasts activated by linked or nonlinked recognition. TNP-ABC* blasts were incubated at 25,000 cells per culture. To each culture, EL-4 SN (1% vol/vol) and IL-1 (5 units/ml) or no cytokines were added. After an additional 4 days, each culture was assessed for growth by pulsing with 1 μCi of [^{3}H]thymidine for 16 hr. Differentiation was assessed by determining IgM anti-TNP PFC on aliquots of the same culture. Results are mean ± SD. Control values were 1,015 ± 270 cpm per culture (linked activation) and 1,725 ± 371 cpm per culture (nonlinked activation). Stimulation index = response with cytokines/response without cytokines.

DISCUSSION

In the present report, we have utilized purified populations of TNP-binding B cells (TNP-ABC) to study the role(s) of antigen, T cells, and cytokines in the induction of B-cell proliferation and differentiation. Four findings have emerged from these studies: (*i*) TNP-ABC do not proliferate or differentiate into PFC when cultured with TD antigen, a source of cytokines, or both. (*ii*) In the presence of carrier-primed T cells and antigen, TNP-ABC can be induced to proliferate. Proliferation is maximal under conditions of linked recognition but also occurs under conditions of nonlinked recognition. (*iii*) TNP-ABC can be induced to differentiate into PFC when they are cultured with carrier-primed T cells under conditions of linked recognition. In contrast, under conditions of nonlinked recognition, differentiation does not occur. (*iv*) After activation by carrier-primed T cells under conditions of linked recognition, the TNP-ABC* blasts can replicate and terminally differentiate in response to cytokines in the absence of both antigen and T cells. TNP-ABC* blasts activated by nonlinked recognition replicate but do not differentiate in response to the same cytokines.

With regard to the failure of TNP-ABC to proliferate in response to TD antigens, previous investigations have shown that TD antigens do not induce the entry of antigen-specific B cells into the cell cycle (20, 23). Furthermore, cell cycling could not be promoted by the addition of T-cell replacement factor (TRF), IL-1, or interleukin 2 (IL-2) (23). Other studies have shown that the addition of soluble TNP-protein antigen to B cells does not increase the precursor frequency of the anti-TNP PFC unless T cells are added to the culture (24). Therefore, as suggested by our studies and others, crosslinking of surface immunoglobulin on the B cell by a TD antigen is necessary but insufficient to render B cells responsive to the growth-promoting activities of cytokines.

Our finding that TNP-ABC proliferate when T cells are added under conditions of nonlinked recognition is consistent with previous reports demonstrating that antigen-primed T cells, or T-cell lines and clones, can induce polyclonal proliferation and differentiation of B cells in the presence of the carrier (25, 26). In our studies, Flu-KLH was 20–35% as effective as TNP-KLH in eliciting a B-cell proliferative response. As has been sug-

gested by others (25, 26), the response to the carrier might be due to nonspecific absorption of the Flu-KLH onto the B-cell surface. Such "coated" B cells might then present antigen to the T cells, resulting in cognative T–B cell contact and stimulation of B cells by the activated T cells. Another possible explanation is that these carrier-primed T cells secrete lymphokines not found in the EL-4 supernatant. Because under conditions of nonlinked recognition the TNP-ABC did not differentiate into PFC, this would suggest that either the B cells which are induced to proliferate by nonlinked and linked recognition are different subsets (27) or that the same subset of cells is activated differently by linked and nonlinked recognition.

In contrast to the present results, there are several reports of the generation of PFC responses after induction by nonlinked recognition (28, 29). There are at least two possibilities to explain these differences: (*i*) The percentage of blasts in the starting cell population (that may have been generated *in vivo* by linked recognition), may be lower in the cells used in our studies compared to those used in past ones; these lower numbers may be insufficient to give a measurable differentiative response. (*ii*) The concentration of antigen is relatively low in our studies compared to those of others. Thus, it could be argued that higher doses of antigen are required by B cells to present antigen to T cells in a way that results in T-cell-mediated induction of B-cell differentiation. Clearly, further studies are needed to elucidate the apparent discrepancy between the results reported here and those described previously.

Classical studies examining H-2-restricted linked recognition suggest that T cells must be physically linked to B cells in order to induce their differentiation (1–5). This observation has been extended to show that maximal B-cell growth also requires linked recognition (19). The present study confirms these findings but also shows that, after the initial antigen-driven, T cell-dependent linked activation event, cytokines can replace the requirement for T cells and antigen in the subsequent induction of B-cell differentiation and proliferation.

Taken together, these results suggest that (*i*) after interaction of resting TNP-ABC with carrier-primed T cells and antigen, the cells enlarge and become responsive to cytokines; (*ii*) TNP-ABC blasts generated under different circumstances (linked and nonlinked) express different cytokine responsiveness. This latter observation has important implications for comparing the effects of cytokines in polyclonal and antigen-driven B-cell systems in which cells have been stimulated by different ligands. Nonetheless, in polyclonal systems, it has been demonstrated that large putatively "activated" cells respond to nonspecific T-cell help (30, 31), whereas resting B cells require major histocompatibility complex-restricted help and crosslinking of surface Ig receptors (31). These results are in agreement with our own studies, which demonstrate that T cells are required for the initial activation step and that cytokines subsequently induce and sustain the differentiation and continued proliferation of the activated B cells.

In summary, the present results emphasize the importance of the nature of the initial signal in determining the potential of the resultant activated B cells. Thus, B-cell blasts generated by linked recognition have the potential for terminal differentiation, provided the appropriate cytokines are supplied, whereas B-cell blasts activated by nonlinked recognition proliferate but do not differentiate in response to the same mixture of cytokines. It is not clear whether B-cell blasts activated by anti-Ig are similar to one or the other type of B-cell blasts characterized in the present studies. It is also not known whether the different potential of these blasts is because they represent different B-cell lineages or whether the nature of the initial signaling can stimulate the same cell type to take different pathways or to stop at different stages of differentiation. Clearly, an answer to this question is important for an understanding of the induction and regulation of antibody formation and immunologic memory.

We thank Ms. M. Thornton for expert technical assistance and Ms. G. Cheek for patient secretarial assistance. We thank Drs. M. Patterson and J. Kettman for expert advice and assistance in the use of the cell analysis facility. We thank Dr. P. Lipsky for helpful comments regarding the manuscript. This work was supported by National Institutes of Health Grants AI-12789 and AI-11851.

1. Mitchison, N. A. (1969) in *Immunological Tolerance*, eds. Landy, M. & Braun, W. (Academic, New York), pp. 149–172.
2. Katz, D. H. (1977) in *Lymphocyte Differentiation, Recognition and Regulation*, ed. Katz, D. (Academic, New York), pp. 382–481.
3. Dutton, R. W., Campbell, P., Chan, E., Hirst, J., Hoffman, H., Kettman, J., Lesley, J., McCarthy, M., Mishell, R. I., Raidt, D. J. & Vann, D. (1971) in *Cellular Interactions in the Immune Response*, eds. Cohen, S., Cudknowicz, G. & McCluskey, R. T. (Karger, Basel, Switzerland), pp. 31–57.
4. Kettman, J. & Dutton, R. W. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 699–704.
5. Bullock, W. W. & Rittenberg, M. B. (1970) *J. Exp. Med.* **132**, 926–940.
6. Howard, M. & Paul, W. E. (1983) *Annu. Rev. Immunol.* **1**, 307–333.
7. Dutton, R. W., Falkoff, R., Hirst, J. A., Hoffman, M., Kappler, J. W., Kettman, J. R., Lesley, J. F. & Vann, D. (1971) *Prog. Immunol.* **1**, 355–389.
8. Schimpl, A. & Wecker, E. (1975) *Transplant. Rev.* **23**, 176–188.
9. Howard, M., Farrar, J., Hilfiker, M., Johnson, B., Takatsu, K., Hamaoka, T. & Paul, W. E. (1982) *J. Exp. Med.* **155**, 914–923.
10. Pure, E., Isakson, P. C., Takatsu, K., Hamaoka, T., Swain, S. L., Dutton, R. W., Dennert, G., Uhr, J. W. & Vitetta, E. S. (1981) *J. Immunol.* **127**, 1953–1958.
11. Pure, E., Isakson, P. C., Kappler, J. W., Marrack, P., Krammer, P. H. & Vitetta, E. S. (1983) *J. Exp. Med.* **157**, 600–612.
12. Takatsu, K., Tanaka, K., Tominga, A., Kumahara, Y. & Hamaoka, T. (1980) *J. Immunol.* **125**, 2646–2653.
13. Melchers, F., Anderson, J., Lernhardt, W. & Schreier, M. H. (1980) *Eur. J. Immunol.* **10**, 679–684.
14. Swain, S. L., Dennert, G., Warner, J. F. & Dutton, R. W. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2517–2521.
15. Isakson, P. C., Pure, E., Vitetta, E. S. & Krammer, P. H. (1982) *J. Exp. Med.* **155**, 734–748.
16. Severinson, E., Bergstedt-Lindquist, S., Van Der Loo, W. & Fernandez, C. (1983) *Immunol. Rev.* **67**, 73–84.
17. Kishimoto, T. & Ishizaka, K. (1975) *J. Immunol.* **114**, 1177–1184.
18. Mayer, L., Fu, S. M. & Kunkel, H. G. (1982) *J. Exp. Med.* **156**, 1860–1870.
19. Snow, E. C., Noelle, R. J., Uhr, J. W. & Vitetta, E. S. (1983) *J. Immunol.* **130**, 614–618.
20. Snow, E. C., Vitetta, E. S. & Uhr, J. W. (1983) *J. Immunol.* **130**, 607–613.
21. Wysocki, L. J. & Sato, V. L. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2844–2848.
22. Farrar, J., Fuller-Farrar, J., Simon, P., Hilfiker, M., Stadler, B. & Farrar, W. (1980) *J. Immunol.* **125**, 2555–2558.
23. Cambier, J. G., Monroe, J. G. & Neale, M. J. (1982) *J. Exp. Med.* **156**, 1635–1643.
24. Keller, D. M., Swierkosz, J. E., Marrack, P. & Kappler, J. W. (1980) *J. Immunol.* **124**, 1350–1359.
25. Tse, H. Y., Mond, J. J. & Paul, W. E. (1981) *J. Exp. Med.* **153**, 871–882.
26. Jones, B. & Janeway, C. A. (1981) *Nature (London)* **292**, 547–550.
27. Asano, Y., Singer, A. & Hodes, R. J. (1981) *J. Exp. Med.* **154**, 1100–1115.
28. Asano, Y. & Hodes, R. J. (1982) *J. Exp. Med.* **155**, 1267–1276.
29. Asano, Y., Shigeta, M., Fathman, C. G., Singer, A. & Hodes, R. J. (1982) *J. Exp. Med.* **156**, 350–360.
30. Andersson, J., Schreier, M. H. & Melchers, F. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1612–1616.
31. Julius, M. H., Vonboehmer, H. & Sidman, C. L. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1989–1993.