

Induction of Immunological Tolerance in Bone Marrow-Derived Lymphocytes of the IgE Antibody Class

(dinitrophenyl poly(Glu,Lys)/passive cutaneous anaphylaxis/adoptive transfer)

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ABSTRACT Administration of the 2,4-dinitrophenyl (Dnp) determinant conjugated to the "nonimmunogenic" copolymer of D-glutamic acid and D-lysine (D-GL) induces Dnp-specific immunological tolerance which is essentially restricted to bone marrow-derived (B) lymphocytes. Previous studies have demonstrated tolerance induction by Dnp-D-GL in predominantly B lymphocytes of the IgG class. The present studies establish and analyze conditions for tolerance induction in Dnp-specific B lymphocytes of the IgE antibody class by taking advantage of: (i) the relatively easy capacity to induce tolerance with Dnp-D-GL in an animal or cell population previously primed to the Dnp determinant; (ii) previously established parameters for the successful adoptive transfer of Dnp-specific IgE antibody responses; and (iii) the capacity to induce, and maintain a state of Dnp-specific tolerance with Dnp-D-GL in adoptive transfer responses. Using these approaches, we have successfully rendered Dnp-specific B cells of the IgE class profoundly and apparently irreversibly tolerant after exposure of such cells to Dnp-D-GL. These observations represent a starting point of wide potential for solving many therapeutic problems concerned with clinical allergies.

The capacity to induce specific immunological tolerance that is essentially restricted to bone marrow-derived (B) lymphocytes of an individual has far-reaching clinical therapeutic potential. The successful induction of true hapten-specific tolerance *in vivo* by several investigators (1-7) may, indeed, reflect such a state of restricted B-cell tolerance (for review, see ref. 8). We have made extensive studies in a model of 2,4-dinitrophenyl (Dnp)-specific tolerance in inbred guinea pigs (1), and more recently in mice (5), in which tolerance was readily induced by treatment of such animals with a "nonimmunogenic" Dnp conjugate of the copolymer of D-glutamic acid and D-lysine (D-GL). Animals treated with Dnp-D-GL manifested profound Dnp-specific tolerance as reflected by their inability to respond to immunization with immunogenic Dnp-protein conjugates. The tolerant state in this model has been clearly shown to be expressed predominantly in the population of Dnp-specific B-lymphocyte precursors of antibody-forming cells, and has therefore been interpreted by us to reflect a central mechanism (1, 5, 8, and see *Discussion*).

The studies cited above (1-7) concerned induction of tolerance in predominantly the IgG and IgM antibody classes.

Abbreviations: T lymphocytes, thymus-derived; B lymphocytes, bone marrow-derived; Dnp, 2,4-dinitrophenyl; D-GL, copolymer of D-glutamic acid and D-lysine; BGG, bovine gamma globulin; ASC, extract of *Ascaris suum*; PCA, passive cutaneous anaphylaxis.

The present studies were designed to establish and analyze conditions for induction of tolerance in specific B lymphocytes of the IgE antibody class. This has been accomplished by taking advantage of: (i) a unique feature of the Dnp-D-GL-tolerance model—it is relatively easy to induce tolerance in an animal or cell population previously primed to the Dnp determinant (1, 5); (ii) previously established parameters for successful adoptive transfer of Dnp-specific IgE antibody responses in mice (9, 10); and (iii) the capacity to induce, and maintain, a state of Dnp-specific tolerance with Dnp-D-GL in adoptive transfer responses of the IgG class (5). Using these approaches, we have successfully rendered Dnp-specific B cells of the IgE class profoundly and apparently irreversibly tolerant after exposure of such cells to Dnp-D-GL.

MATERIALS AND METHODS

Proteins and Hapten-Protein Conjugates. The copolymer of D-glutamic acid and D-lysine (D-GL) was obtained from Pilot Chemicals, Inc., Watertown, Mass. The polymer had an average molecular weight of 55,000 and a ratio of glutamic acid to lysine residues of 60:40. *Ascaris* proteins (ASC), extracted from *Ascaris suum* (11), were kindly supplied by Dr. Kurt J. Bloch. Bovine gamma globulin (BGG) was obtained from Pentex Biochemicals, Kankakee, Ill. The following 2,4-dinitrophenyl (Dnp) conjugates were prepared as described (12, 13): Dnp_{2.1}-ASC, and Dnp₃₂-BGG. Preparation of Dnp₃₂-D-GL has been described in detail (1). Subscripts refer to the number of moles of Dnp per mol of carrier for BGG and D-GL and mol of Dnp $\times 10^{-7}$ per mg of ASC.

Animals. Mice of the inbred lines A/J and SJL were obtained from the Jackson Laboratories, Bar Harbor, Me. Random-bred white female CFW mice were obtained from Carworth Farms, New City, N.Y. All mice were immunized or used as recipients at 8-12 weeks of age.

Immunizations and Adoptive Cell Transfers. Mice used as donors of primed spleen cells were immunized by intraperitoneal injection of 10 μ g of Dnp-ASC mixed with 4 mg of Al(OH)₃ gel in a total volume of 0.5 ml of saline. Al(OH)₃ gel (alum) was prepared by mixing equal volumes of 2 N Al₂(SO₄)₃ and 2 N NaOH (14). Mixtures of hapten-protein conjugates with alum were prepared immediately before use. At appropriate times after priming and treatment with Dnp-D-GL, the primed donor mice were killed and their spleens removed. Single-cell suspensions in Eagle's minimum essential

medium were prepared, washed, and transferred intravenously to syngeneic irradiated (500 R) recipients. Usually 50×10^6 cells were administered to each recipient. Immediately after cell transfer, secondary challenge was performed intraperitoneally with 10 μ g of Dnp-ASC mixed with 2 mg of alum. All recipient mice were bled from the retroorbital plexus 7 days after cell transfer and challenge, and serum anti-Dnp antibody levels were determined as described below.

Measurement of Anti-Dnp Antibodies. (a) *IgE antibodies:* The concentration of reaginic (IgE) anti-Dnp antibodies in pools of sera from groups of five mice were determined by passive cutaneous anaphylaxis (PCA) reactions with shaven CFW or SJL test mice (14, 15). Briefly, 25- μ l samples of serum from each mouse within a given group were pooled and serially diluted (2-fold) in saline. 50 μ l of each serum dilution was injected intradermally into the dorsal skin surface of the test mice. 48 hr after intradermal sensitization, Dnp-specific PCA reactions were elicited by intravenous injection of 5, 50, or 500 μ g of Dnp-BGG in 0.3 ml of 0.5% Evans blue dye dissolved in 5% dextrose-water solution. Reactions were read and recorded as the reciprocal of the highest dilution of serum evoking threshold PCA reactivity (5-mm diameter). Sera that failed to elicit detectable PCA reactions were arbitrarily assigned a value of <5. It is well-established that homocytotropic antibodies detected by this procedure are of the IgE class (16). Details of their Dnp specificity have been described (9).

(b) *IgG serum antibodies:* Serum IgG anti-Dnp antibody levels were determined in individual sera by a modified Farr technique (12, 17) using [^{3} H]Dnp- ϵ -amino-*n*-caproic acid as described (12). With standard curves constructed from specific anti-Dnp antibody of corresponding strains of mice, percentage of binding was converted into amount of specific anti-Dnp antibody in μ g/ml of serum. For statistical analysis, antibody levels of individual animals were logarithmically transformed and geometric means and standard errors were calculated. In those mice in which no significant antibody could be detected in the serum, a value of 0.10 μ g/ml was arbitrarily assigned to allow logarithmic transformation of the data. Group comparisons were made employing Student's *t*-test.

TABLE 1. Induction of Dnp-specific tolerance in both IgE and IgG antibody classes by pretreatment of A/J mice with Dnp-D-GL

Protocol			Serum anti-Dnp antibody	
Group	Pretreatment	Days after priming	IgE (PCA)*	IgG (μ g/ml)†
A	None	13	320	79.2
		20	320	212.6
B	Dnp-D-GL (500 μ g $\times$ 2)	13	40	1.4
		20	20	8.7

* Data are expressed as the reciprocal of the highest PCA titer elicited by pooled sera from 7 mice of each group bled on days 13 and 20 after primary immunization with Dnp-ASC. PCA reactions were elicited with a test dose of 500 μ g of Dnp-BGG.

† Data are expressed as geometric mean anti-Dnp antibody levels of sera from individual mice in each group of seven mice bled on days 13 and 20 after primary immunization.

TABLE 2. Preferential depression of high-affinity IgE antibody-forming cells

Group	PCA titer elicited with Dnp-BGG		
	500 μ g	50 μ g	5 μ g
Untreated	640	640	640
Tolerant	40	10	<5

Sera analyzed were the same as those presented in the top panel of Fig. 2. Doses of Dnp-BGG varying over 2 logs as indicated were used as test antigen in eliciting PCA reactions.

RESULTS

Induction of Dnp-Specific Tolerance in Both IgE and IgG Antibody Classes with Dnp-D-GL When Treatment Precedes Primary Immunization. Normal A/J mice received two injections on successive days of 500 μ g of Dnp-D-GL intraperitoneally in saline. 1 Week later, these mice and a group of untreated control mice were primarily immunized with 10 μ g of Dnp-ASC mixed with 4 mg of alum. This immunization procedure induces good IgE and IgG primary anti-Dnp antibody responses in such mice (9). All animals were bled on days 13 and 20 after priming and their sera analyzed for anti-Dnp antibodies.

As shown in Table 1, untreated control mice in group A manifested significant IgE and IgG anti-Dnp antibody levels on days 13 and 20 after primary immunization with Dnp-ASC. Previous studies have documented that the peak IgE antibody titer is reached on day 20 after priming in this fashion (9). In contrast, mice pretreated with Dnp-D-GL before primary immunization (group B) developed significantly lower anti-Dnp antibody responses in both IgE and IgG antibody classes. It is notable that the degree of suppression resulting from such pretreatment appears to be somewhat greater in the IgG than in the IgE antibody class. However, this finding probably reflects the fact that in this experiment PCA reactions were elicited with a very high dose (500 μ g) of test antigen (Dnp-BGG), thus allowing detection of both low- and high-affinity antibody molecules. The conditions of the Farr assay used to measure IgG antibodies, on the other hand, are such that predominantly moderate-to-high affinity molecules are detected. Since recent studies have demonstrated a preferential depression of the response of high-affinity IgG anti-Dnp antibody in the Dnp-D-GL model (18), it is probable that the results obtained reflect these differences in affinity. This question is investigated in more detail in a subsequent section below.

Induction of Dnp-Specific Tolerance with Dnp-D-GL in an Adoptive Cell Transfer System. The relative ease with which Dnp-specific tolerance can be induced by Dnp-D-GL in a previously immunized animal (1) enabled us to develop a system for induction of tolerance in an adoptive transfer system in mice (5). In the latter studies we were dealing with anti-Dnp responses of the IgG antibody class (5). The more recent development in our laboratory of a system that uses suitably primed donor cells (9, 10) for successful adoptive transfer into irradiated recipients of IgE antibody responses permitted the adaptation of such a model to the study of Dnp-specific tolerance induction in IgE B cells. The advantage

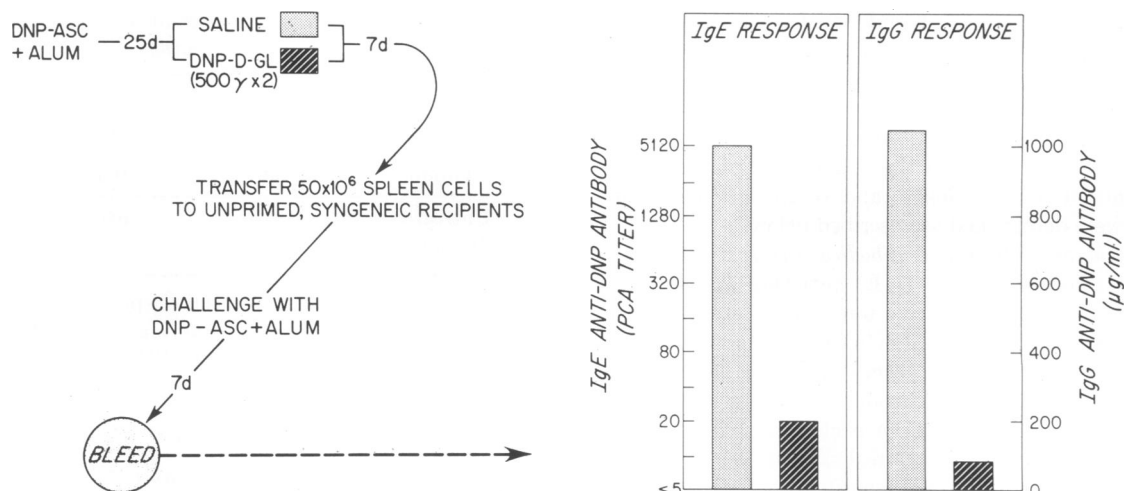


FIG. 1. Maintenance of Dnp-specific tolerance after adoptive transfer of cells from donors treated with Dnp-D-GL. A/J mice were treated per protocol described on left of figure. The serum IgE and IgG anti-Dnp antibody responses of groups of five mice on day 7 after secondary challenge are illustrated on the right. Statistical comparison of the IgG responses of recipients of control donor cells and cells from donors treated with Dnp-D-GL yielded $P < 0.001$. d, days; γ , μg .

to such a system is the obviation of any contribution of circulating antibodies present in a primed intact animal, and the ability to delineate the longevity of the tolerant state by using serial cell transfer techniques.

In the first experiment of this type (Fig. 1), A/J mice were primed with 10 μg of Dnp-ASC plus alum and then treated 25 days later with two successive daily injections of 500 μg of Dnp-D-GL intraperitoneally. Control mice received saline rather than Dnp-D-GL. 1 Week later, pooled spleen cells from the treated or untreated groups were adoptively transferred into syngeneic, unprimed, irradiated recipients which were then secondarily challenged with Dnp-ASC plus alum. As shown in Fig. 1, recipients of cells from untreated donor mice developed high levels of anti-Dnp antibodies of both IgE and IgG classes by day 7 after challenge. On the other hand, recipients of spleen cells from donors treated with Dnp-D-GL manifested marked suppression of antibody production in the IgE class (99%) and in the IgG class (92%). The PCA reactions in this and the following experiment were elicited with a low dose (5 μg) of test antigen (Dnp-BGG).

A second experiment (Fig. 2) was designed to determine to what extent secondary challenge in donor mice before cell transfer would affect the tolerant state demonstrable in adoptive cell recipients. Thus, 25 days after priming, A/J mice primed with Dnp-ASC were either treated or not with Dnp-D-GL as in the preceding experiment. 1 Week later, these two groups of mice were secondarily challenged with Dnp-ASC. 7 Days thereafter all mice were bled, and their spleen cells were removed and transferred to groups of irradiated syngeneic recipients. All recipients were given a final challenge with Dnp-ASC plus alum and then bled 7 days later.

The top panel of Fig. 2 illustrates the anti-Dnp antibody levels in the two groups of donor mice on day 7 after secondary challenge. Note the virtual abrogation of both IgE and IgG antibody responses in mice treated with Dnp-D-GL as contrasted to the untreated controls. The maintenance of this tolerant state after adoptive transfer of such cells to irradiated recipients is depicted in the bottom panel of Fig. 2, thus corroborating the results of the preceding experiment (Fig. 1). The amount of IgE antibody in the irradiated

recipients of tolerant donor cells was slightly higher than in the tolerant donors. This finding probably reflects a combination of nonspecific proliferative stimuli in an irradiated environment, and the absence of circulating Dnp-D-GL, which may bind to a small proportion of residual anti-Dnp antibodies in the tolerant donors.

Preferential Induction of Tolerance in High-Affinity IgE B Cells. Induction of tolerance with Dnp-D-GL in the IgG antibody class in guinea pigs results in a preferential depression of the high-affinity antibody-forming cells (18). We have examined this question for IgE antibody-forming cells in the present studies by using various doses of test antigen for elicitation of PCA reactions with sera from groups of mice that were either treated with Dnp-D-GL or not. Representative results in Table 2 demonstrate that small but definite differences in PCA titers occur with sera from tolerant animals, depending on the dose of test antigen used in the PCA test. Thus, somewhat higher titers were obtained with an excessive amount (500 μg) of Dnp-BGG than with the lower doses in testing sera from tolerant mice, whereas no such difference in titer was observed with sera from untreated mice. It is probable that the differences seen with tolerant sera reflect the presence of antibodies of predominantly low average affinity which are more likely to be detected at high rather than low test antigen doses.

DISCUSSION

In the studies reported here, we have shown that administration of the Dnp derivative of the copolymer of D-glutamic acid and D-lysine (D-GL) to mice induces a state of Dnp-specific tolerance in the IgE as well as the IgG antibody class, irrespective of the immune status of such animals at the time of treatment. These results extend our previous observations on Dnp-specific tolerance in inbred guinea pigs and mice. These studies were concerned with antibody responses predominantly of the IgG class (1, 5). We have focused our present efforts on induction of tolerance in the IgE antibody-forming cells and their precursors because of the potential clinical therapeutic applications of this model.

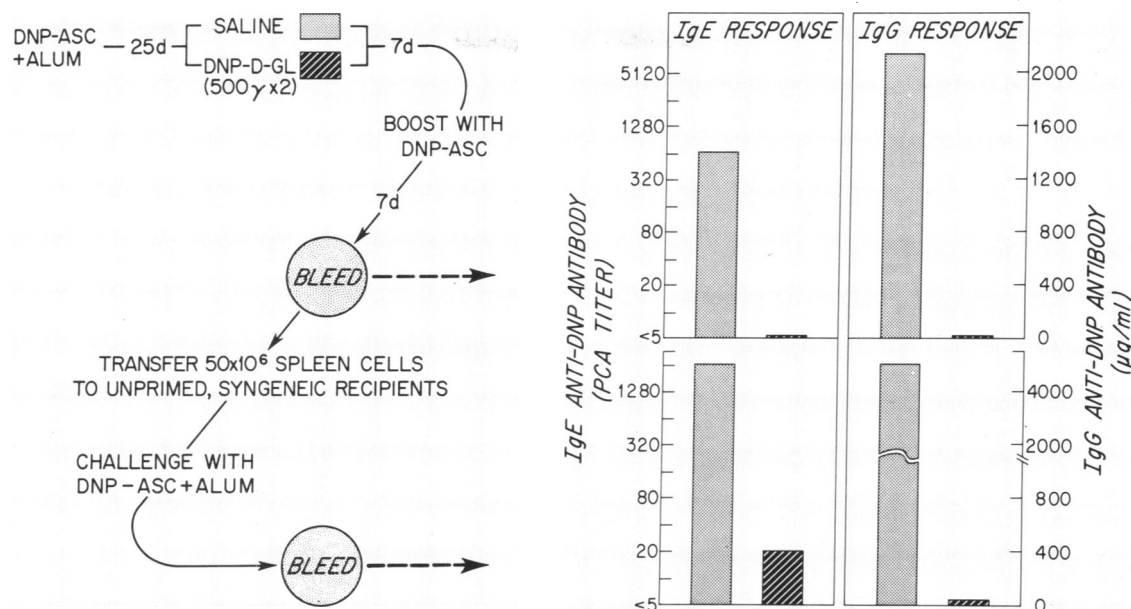


FIG. 2. Protocol depicted on left of figure. The serum IgE and IgG anti-Dnp antibody responses of the donors of spleen cells (top right panel) and recipients of those cells (bottom right panel) on day 7 after challenge are illustrated. Each group consisted of five mice. Statistical comparisons of the IgG responses of donors and recipients of control cells with cells treated with Dnp-D-GL yielded $P < 0.001$ in all cases.

Using a priming regimen designed to elicit very good anti-Dnp antibody responses of the IgE as well as the IgG class, and a system recently developed by us which permits adoptive transfer of IgE antibody responses into irradiated recipient mice (9, 10); we have been able to induce Dnp-specific tolerance in the IgE antibody-forming cell population of either unprimed or previously primed mice. Thus, treatment of unprimed mice with appropriate doses of Dnp-D-GL 1 week before primary immunization with Dnp-ASC markedly diminished the primary anti-Dnp antibody response in both the IgE and IgG classes as compared to controls (Table 1). The capacity to induce Dnp-specific tolerance in an already primed population was demonstrated in experiments using adoptively transferred primed spleen cells (Figs. 1 and 2). In these experiments, cells from primed donor mice that had been treated with Dnp-D-GL *in situ* 1 week before transfer to irradiated recipients were unable to develop adoptive secondary anti-Dnp responses of either IgE or IgG antibody classes. The degree of unresponsiveness as compared to controls was 92–100% in both classes. Moreover, the tolerant state induced in the donor cell populations was not diminished by administering to donor mice an immunogenic dose of Dnp-ASC as an intervening secondary challenge between exposure to Dnp-D-GL and subsequent transfer to irradiated recipients (Fig. 2).

We have previously presented detailed evidence in the Dnp-D-GL model for a central mechanism of tolerance which is expressed predominantly in the population of Dnp-specific antibody-forming cells and their precursors, i.e., B lymphocytes. This conclusion is derived from the following findings:

(i) The tolerance induced by Dnp-D-GL is hapten-specific and the frequency of both antigen-binding lymphocytes specific for Dnp and cells forming antibodies to Dnp is significantly lower in tolerant animals than in normal or immune animals (1, 18).

(ii) A very marked depression both in plaque-forming cells secreting high-affinity anti-Dnp antibody and in serum antibody of high affinity for Dnp has previously been shown

to exist in IgG class responses in this model, indicating a preferential induction of tolerance in precursor cells bearing high-affinity receptors (18). Evidence obtained in the present studies indicates that this is also the case in B lymphocytes of the IgE antibody class (Table 2).

(iii) The absence of T-cell participation in the process of tolerance induction by Dnp-D-GL has been shown conclusively in very recent *in vitro* studies using lymphocytes from congenitally athymic (nude) mice (19).

Recent studies from our laboratory designed to probe the questions of mechanism of induction of tolerance and fate of tolerant cells in the Dnp-D-GL model led us to conclude that the Dnp-specific tolerance induced in this way is an example of irreversible inhibition of cell reactivity to antigen reflecting yet-to-be-determined events at the intra- and sub-cellular level (5). These intracellular events, we believe, are the ultimate and apparently irrevocable (for a given cell) consequence of a tolerogenic signal generated at the cell-surface membrane after binding of Dnp-D-GL to specific immunoglobulin receptors (there is no reason to believe that it is necessary for Dnp-D-GL to enter the cell in order for induction of tolerance to occur). These conclusions were based on observations on the prolonged maintenance of the tolerant state through as many as two serial adoptive transfers performed over a period of time of at least 24 days from the single exposure of such cells to the tolerogen. More conclusively to the point was the failure of enzymatic treatment with trypsin to reverse the tolerant state induced by *in vitro* exposure of primed cells to Dnp-D-GL (5). Moreover, cells cannot be "rescued" from the tolerogenic signal induced by *prior* exposure to Dnp-D-GL by providing a nonspecific T-cell activation caused by a graft-versus-host reaction, i.e., allogeneic effect (1, 8; Hamaoka, T., Katz, D. H. & Benacerraf, B., manuscript in preparation). In contrast, exposure of Dnp-specific lymphocytes to Dnp-D-GL *during* the course of an allogeneic effect results in the development of immunity rather than tolerance to this molecule (1, 8, 20–22). This ob-

servation indicates that during a short time after the binding of the tolerogenic molecule Dnp-D-Gl, the specific B lymphocytes are activated and therefore susceptible to induction for differentiation and antibody production provided an appropriate T-cell signal is exerted. This period of activation is, however, extremely short and is followed by complete refractoriness.

Although the above findings and conclusions derive from studies on IgG antibody class responses, there is no evidence to suggest that similar (or identical) molecular events do not also occur in the induction of tolerance in B lymphocytes of the IgE class. Indeed, certain features such as the capacity to induce tolerance in previously primed lymphocytes and maintenance of unresponsiveness after adoptive transfer have been shown in the present studies to occur for both antibody classes. Nevertheless, even if there are slight mechanistic differences between the two classes, the observations reported here represent the first demonstration of restricted B-cell tolerance of the IgE antibody class, and provide therefore a starting point for many areas of experimentation ultimately destined to solve many therapeutic problems concerning clinically allergic patients.

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1. Katz, D. H., Davie, J. M., Paul, W. E. & Benacerraf, B. (1971) *J. Exp. Med.* **134**, 201-223.
2. Havas, H. F. (1969) *Immunology* **17**, 819-830.
3. Borel, Y. (1971) *Nature New Biol.* **230**, 180-182.
4. Golan, D. T. & Borel, Y. (1971) *J. Exp. Med.* **134**, 1046-1061.
5. Katz, D. H., Hamaoka, T. & Benacerraf, B. (1972) *J. Exp. Med.* **136**, 1404-1429.
6. Cohen, B., Davie, J. M. & Paul, W. E. (1973) *J. Immunol.* **110**, 213-218.
7. Hamilton, J. A. & Miller, J. F. A. P. (1973) *Eur. J. Immunol.*, in press.
8. Katz, D. H. & Benacerraf, B. (1972) *Advan. Immunol.* **15**, 1-93.
9. Hamaoka, T., Katz, D. H., Bloch, K. J. & Benacerraf, B. (1973) *J. Exp. Med.* **138**, 306-311.
10. Hamaoka, T., Katz, D. H. & Benacerraf, B. (1973) *J. Exp. Med.* **138**, 538-556.
11. Strejan, G. & Campbell, D. H. (1967) *J. Immunol.* **98**, 893-899.
12. Katz, D. H., Paul, W. E., Goidl, E. A. & Benacerraf, B. (1970) *J. Exp. Med.* **132**, 261-282.
13. Benacerraf, B. & Levine, B. B. (1962) *J. Exp. Med.* **115**, 1023-1036.
14. Levine, B. B. & Vaz, N. M. (1970) *Int. Arch. Allergy Appl. Immunol.* **39**, 156-166.
15. Ovary, Z. (1958) *J. Immunol.* **81**, 355-357.
16. Bloch, K. J. (1967) *Progr. Allergy* **10**, 84-131.
17. Farr, R. S. (1958) *J. Infec. Dis.* **103**, 329-335.
18. Davie, J. M., Paul, W. E., Katz, D. H. & Benacerraf, B. (1972) *J. Exp. Med.* **136**, 426-438.
19. Nossal, G. J. V., Pike, B. & Katz, D. H. (1973) *J. Exp. Med.* **138**, 312-317.
20. Katz, D. H. (1972) *Transplant. Rev.* **12**, 141-179.
21. Osborne, D. P., Jr. & Katz, D. H. (1972) *J. Exp. Med.* **136**, 439-454.
22. Osborne, D. P., Jr. & Katz, D. H. (1973) *J. Exp. Med.* **137**, 991-1008.