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Significance of Cell Interactions in Production of Graft Versus Host Splenomegaly[1]

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INTRODUCTION

Synergistic interactions between two different types of immunologically competent lymphocytes, thymus-derived (T cells) and bone marrow-derived (B cells), have been shown to occur in humoral immune responses(1–4). Whether such interactions take place in cell mediated immune responses is less clear. Recently, a number of reports have described synergistic reactions between different lymphoid cell populations in the production of graft versus host (GVH) disease(5–8) and in the production of delayed hypersensitivity lesions(9–11), two forms of cellular immunity. In only one case, however, was there evidence that both cell populations had immunologic specificity(6). In that case, neither of the interacting cell populations were B cells.

It is well-known that in the reactions of delayed hypersensitivity, lymphocyte (almost assuredly T cell): macrophage interactions take place in which the role of the macrophage is nonspecific(12). That macrophages are derived from the bone marrow has also been well demonstrated(12,13). Furthermore, in GVH reactions it has been well demonstrated that foreign lymphocytes (in this case it has been shown that these are T cells)(14–19) interact with host cells (some of which have been shown to come from the bone marrow) in the production of the splenomegaly which is associated with that disease. This has been interpreted to mean that the B cell in this case fulfills a role similar to that seen in delayed hypersensitivity lesions, in that it is nonspecific(8). However, it is alternatively possible that the foreign T cell acts as an antigen recognition cell that activates

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B cells to perform autoimmune reactions. For example, it has been shown that tolerant animals have B cells which are quite capable of making antibodies against the tolerated antigen if they are activated by nontolerant T cells(20–21). The possibility that a similar situation could exist in GVH reactions is strengthened by the observation that these reactions are much easier to produce in newborn animals than in adults(22). Newborn animals are relatively immunologically incompetent, and therefore it may be that their B cells have not yet acquired tolerance, and thus are more easily activated. Similarly, sublethal irradiation which enhances GVH splenomegaly in adult recipients(22,23) is well-known to abrogate tolerance(24).

We report herein on a number of different attempts to demonstrate autoimmune activity on the part of the B cell, all of which were negative. We have done this by making secondary transfers of spleens in which T:B cell interaction took place to see if this produced increased immunocompetence of the cells in the spleen undergoing GVH reactions. In addition, we have immunized mice in the presence of T cells and then removed their T cells by use of an anti-theta serum to see if any immunologic competence was then present in the B cell population. Our results suggest that most, if not all, interactions between T and B cells in GVH splenomegaly are immunologically nonspecific, or not concerned with the generation of cells with GVH activity.

MATERIALS AND METHODS

Mice. All mice were obtained from the Jackson Laboratories, Bar Harbor, Maine. They were kept in our animal colony for at least 1 week after shipment. Recipient mice were male C3D2F1 mice (C3H/HE × DBA/2). Parental donor mice were male C3H mice. C3H(H2K) and DBA(H2D) mice differ at the H2 locus and cause a reliable GVH reaction(7,22).

Cell suspensions. Spleen, lymph node and thymus cell suspensions were made by cutting the fresh organs into small pieces and gently squashing them between sterile glass slides in ice-cold medium M199. The cells were washed twice before use and were counted in a hemocytometer using Trypan blue dye exclusion method as a test of viability. All cell suspensions were introduced via tail vein injections. Cell dosages varied from 5×10^5 to 8×10^7 cells per mouse delivered in a constant 0.2 ml.

Irradiation. All irradiation was performed with a Siemans Stabillipan 250 kV X-ray machine at a dose rate of 85 R p/min. All CDF1 recipients received 350 R. Sublethal irradiation has been shown to enhance the expression of GVH disease among adult mice(22,23).

Preparation of bone marrow reconstituted mice. Bone marrow cell suspensions were made by washing out the femurs of 8-week-old C3H mice with ice-cold medium M199. C3H recipients were thymectomized at 7 weeks. One week later, these same mice received 850 R (central axis dose) whole body irradiation. Within a few hours they were given 5×10^6 viable syngeneic bone marrow cells via

tail vein injection. The spleens of these animals served as a source of B cells. However, since cells other than B cells were present in the bone marrow transferred, we shall refer to the entire population as bone marrow-derived cells (BMDC).

Preparation of anti-theta serum. This anti-serum was raised by the technique of Reif(25). Seven weekly injections of 1×10^7 C3H thymocytes were made into AKR mice. One week later these mice were exsanguinated and their serum separated from the blood. Antibodies were titrated for cytotoxicity using Trypan blue dye exclusion as a measure of cell viability. Ninety-eight percent kill was obtained when one part anti-serum (diluted 1:20) was mixed with one part C3H thymocytes (1×10^7/cc) and one part guinea pig complement.

Skin graft chimeras. C3H adults were thymectomized, lethally irradiated 1 week later and immediately reconstituted with 5×10^6 syngeneic bone marrow cells. Fresh skin from adult DBA mice was cut into squares, 1.5×1.5 cm and grafted to the left flank of C3H adult chimeras after the method of Billingham and Medawar(26). They were examined daily for signs of rejection.

Assay of graft versus host activity. Spleens were harvested on day 14 as previous studies had shown this was the time of maximal splenomegaly(7). They were weighed as were the mice from which they came and the results are expressed in terms of spleen/body weight ratio.

Statistical analysis. All statistical analyses were performed using Student's t test.

RESULTS

The first experiment we report tests the requirement for foreignness of the two cell populations (T cells and BMDC) in the synergistic production of GVH splenomegaly. In this experiment 7-week-old CDF_1 mice were given 350 R of X-irradiation and then were inoculated with either parental (C3H) or syngeneic (CDF_1) thymocytes; parental or syngeneic BMDC; or a combination of thymocytes and BMDC in a 1:1 ratio. All mice received the same total of cells (3×10^7).

The results (Table 1) show that significant splenomegaly was produced only when parental thymocytes were inoculated and then only when BMDC were added. Both the parental ($P<0.01$) and the syngeneic ($P<0.005$) BMDC were able to produce the splenomegaly in cooperation with the parental thymocytes. As had been previously noted, the parental thymocytes when inoculated alone, produced spleens that were significantly smaller than those of uninoculated controls ($P<0.005$)(7).

Since isologous BMDC synergized as well as parental BMDC with parental thymocytes, we performed several experiments to see whether other types of syngeneic lymphoid tissue could also syngergize with parental thymocytes.

First isologous spleens were compared with parental BMDC. The spleen cell population contains T cells and therefore this experiment tests whether or not the presence of syngeneic T cells affects the synergy. The results (Table 2) show that the isologous spleen cells were able to synergize ($P<0.001$) as well or perhaps

even better than were the parental BMDC ($P<0.001$). (All recipient mice received a total of 4×10^7 cells; cell combinations were in a 1:1 ratio.) As previously noted, giving thymocytes without BMDC or spleen cells resulted in the production of significantly smaller spleens ($P<0.001$).

In the next experiment a comparison was made between the capacity of spleen cells from adult (7 week old) and newborn spleen cells to synergize with parental thymocytes. Adult spleen cells contain a much higher proportion of immunologically competent cells and therefore should the isologous population require immunologic competence to enact synergy, the adult cell population should synergize better. All mice (except the radiation controls) received a total of 3×10^7 cells in this experiment (again cell combinations were in a 1:1 ratio). The results (Table 3) show that both adult ($P<0.005$) and newborn ($P<0.005$) spleen cells synergized with parental thymocytes equally well. As noted before, parental thymocytes given alone produced significantly smaller spleens ($P<0.001$).

In the next experiment a comparison was made between syngeneic lymph node cells and syngeneic bone marrow cells in their ability to synergize with the parental thymocytes in the production of splenomegaly. This experiment differed

TABLE 1

INTERACTION OF DIFFERENT THYMOCYTE AND BMDC POPULATIONS IN GVH REACTIONS: REQUIREMENT FOR FOREIGNNESS OF CELLS

Origin of cells inoculated		Number of mice	Spleen index[a] $\times 10^{-5}$ (± SD)
Thymocytes	BMDC		
C3H	0	5	289 ± 58
C3H	C3H	8	563 ± 77
C3H	CDF_1	8	508 ± 88
CDF_1	0	5	403 ± 39
CDF_1	C3H	11	382 ± 47
CDF_1	CDF_1	7	429 ± 49
0	0	10	395 ± 51
0	C3H	5	417 ± 75
0	CDF_1	5	344 ± 28

[a] Spleen/body weight ratio on day 14. All recipient CDF_1 mice got 350 R.

TABLE 2

COMPARISON OF PARENTAL BMDC WITH ISOLOGOUS SPLEEN CELLS IN THE ABILITY TO INTERACT WITH PARENTAL THYMOCYTES IN GVH REACTIONS

Cells inoculated		Number of mice	Spleen Index[a] $\times 10^{-5}$ (± SD)
Thymocytes	Other		
C3H	0	6	196 ± 35
C3H	C3H BMDC	6	596 ± 80
C3H	CDF_1 Spleen	6	733 ± 106
0	0	6	449 ± 51
0	C3H BMDC	6	367 ± 16
0	CDF_1 Spleen	6	336 ± 17

[a] Spleen/body weight ratio on day 14. All recipient CDF_1 mice got 350 R.

somewhat from the previous experiments in that instead of controlling for the total cell inoculum as had previously been done, we controlled for the numbers of each cell population. Thus, animals getting two different cell populations received a total of twice as many cells (3×10^7) as animals getting a single type of cell (1.5×10^7). The results (Table 4) differ from those previously noted. In this instance, thymocytes alone produced significant splenomegaly ($P<0.001$). Indeed animals getting only parental thymocytes had the largest spleens. Combining the parental thymocytes with isologous bone marrow also resulted in splenomegaly when compared to animals which got either no cells ($P<0.01$) or bone marrow cells ($P<0.005$) alone. However, the animals getting parental thymocytes and isologous lymph node cells had spleens that were no larger than the animals that got the lymph node cells alone. Thus, in this experiment the lymph node cells had an antagonistic effect on the splenomegaly produced by the parental thymocytes. The fact that thymocytes alone in this instance produced splenomegaly complicated the interpretation of these results. However, it is clear that the addition of lymph node cells which contain a much greater number of immunologically competent cells than do bone marrow cells did not increase the splenomegaly. Indeed the opposite was true; the lymph node cells decreased the splenomegaly produced by parental thymocytes ($P<0.025$) more than the bone marrow cells (P = N.S.). The significance of this is taken up in the discussion.

TABLE 3

COMPARISON OF NEWBORN WITH ADULT ISOLOGOUS SPLEEN CELLS IN THE ABILITY TO INTERACT WITH PARENTAL THYMOCYTES IN GVH REACTIONS

Cells inoculated		Number of	Spleen Index[a] $\times 10^{-5}$
Thymocytes	Spleen	mice	(± SD)
C3H	0	5	300 ± 13
C3H	Adult	7	644 ± 161
C3H	Newborn	7	657 ± 103
0	0	5	411 ± 22
0	Adult	5	372 ± 34
0	Newborn	5	439 ± 56

[a] Spleen/body weight ratio on day 14. All recipient CDF_1 mice got 350 R.

TABLE 4

COMPARISON OF ISOLOGOUS LYMPH NODE WITH ISOLOGOUS BONE MARROW CELLS IN THE ABILITY TO INTERACT WITH PARENTAL THYMOCYTES IN GVH REACTIONS

Cells inoculated		Number of	Spleen Index[a] $\times 10^{-5}$
Thymocytes	Other	mice	(± SD)
C3H	0	7	585 ± 132
C3H	Lymph node	5	397 ± 33
C3H	Bone marrow	7	489 ± 111
0	0	5	325 ± 19
0	Lymph node	8	394 ± 57
0	Bone marrow	8	332 ± 19

[a] Spleen/body weight ratio on day 14. All recipient CDF_1 mice got 350 R.

These experiments indicate that the presence of increased numbers of syngeneic immunologically competent lymphocytes, either T or B, does not increase the splenomegaly resultant from their interaction with foreign thymocytes. In the following experiment we tried to demonstrate some immunologic role for the syngeneic cells by another means.

We reasoned that if the increased spleen weight reflected a generation of immunologically competent cells, then these cells might be able to produce GVH splenomegaly on their own upon transfer to another recipient. Thus, we gave two groups of CDF_1 mice 350 R of X-irradiation. One group was then inoculated with 1.5×10^7 parental thymocytes and the other group received the same number of parental thymocytes plus 1.5×10^7 isologous spleen cells. Two weeks later, the spleens were harvested and weighed; spleen cell suspensions were then made and reinoculated into other sublethally irradiated F_1 recipients. Three different concentrations of spleen cells were given to eight mice each. There were: 1.5×10^7 cells, a 1:3 dilution of that and a further 1:3 dilution. These secondary recipients were killed 2 weeks later and their spleen body weight ratios were determined.

There was no significant difference in results between the groups getting the three concentrations and therefore the pooled data are presented in Fig. 1. It can be seen that, similar to the last reported experiment and in contrast to the earlier experiments, primary recipients which received parental thymocytes alone had more splenomegaly than those that got the thymocytes plus isologous spleen cells ($P<0.025$). Secondary transfers of equivalent numbers of cells from both groups of mice however, produced similar splenomegaly on retransfer. The amount of splenomegaly was quite small, but statistically significant ($P<.001$). If there was any difference at all between the two groups it was that the cells from the mice with smaller spleens produced more splenomegaly on secondary transfer. Thus this experiment failed to reveal any increased immunologic competence as the result of the increased cell proliferation in spleens of mice undergoing a GVH reaction.

We, therefore, made a different attempt to see if we could demonstrate any immunologic competence in BMDC in the production of GVH. To do this, we took 26 C3H mice and deprived them of T cells by thymectomy, lethal irradiation and bone marrow grafting. Half of these were reconstituted with 3×10^7 syngeneic thymocytes. All mice were then given a DBA skin graft and observed for 2 months. The thymocyte-reconstituted mice all rejected their skin grafts with a mean rejection time of 16.3 days. Eight of 13 mice from the unreconstituted group rejected their skin grafts with a mean rejection time of 37.3 days. At this time, all the mice were killed and spleen cell suspensions were made. Some of these cells, from both groups of mice, were then treated with an anti-theta serum and guinea pig complement. Controls were incubated with complement alone. After treatment the cells were inoculated into CDF_1 mice that had received 350 R of X-irradiation. Two weeks later the recipients were killed and spleen/body weight ratios determined (Fig. 2).

The results show that spleen cells from both groups of animals were able to

produce significant splenomegaly. The splenomegaly was not significantly greater with the spleen cells from animals which had been reconstituted with thymocytes. However, in both groups of animals the splenomegaly was significantly reduced by treating the spleen cells with the anti-theta anti-serum. Thus, although cells from putatively thymus-deprived mice could produce GVH splenomegaly after they were immunized to the antigens in the F_1 host, at least a significant portion of them contained the theta antigen, and therefore were most likely T cells which had not been eliminated by our thymus-deprivation(27).

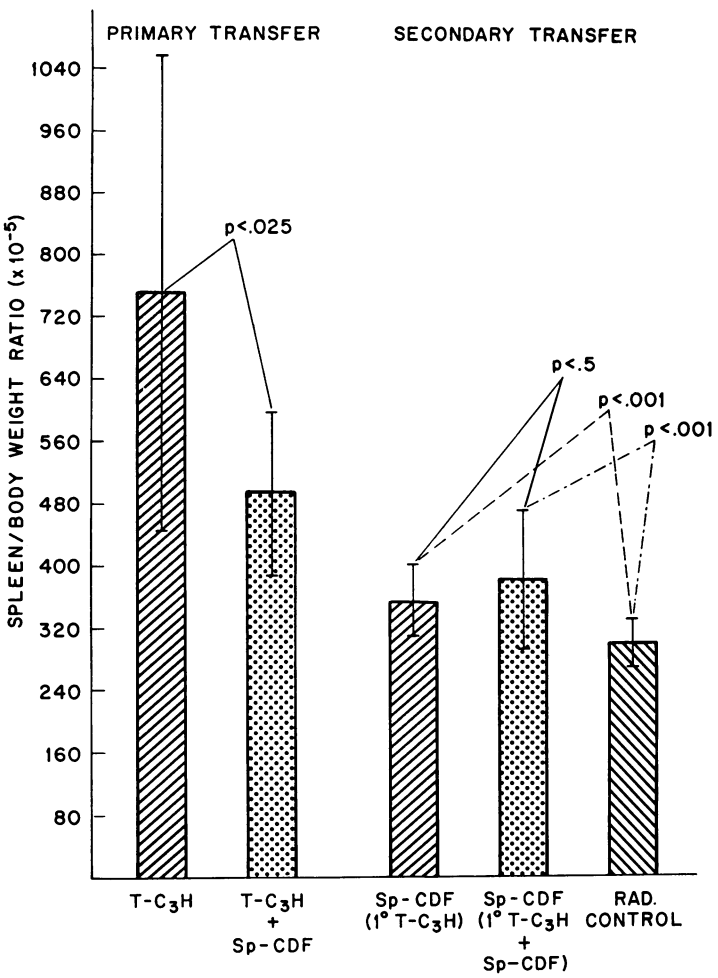

FIG. 1. Spleen indices of sublethally irradiated CDF_1 mice given either parental thymocytes alone (T-C3H) or in combination with isologous spleen cells (T-C3H + Sp-CDF) 14 days previously and of secondary sublethally irradiated CDF_1 recipients getting spleen cells from one or the other or neither of the above two groups.

DISCUSSION

The experiments reported were designed primarily to test the possibility that some form of cell-mediated autoimmune potential was conveyed to syngeneic (F_1) cells when they were induced to proliferate by parental thymocytes in GVH situations. In the course of these studies we confirmed previous reports that thymocytes and BMDC can synergize in the production of GVH splenomegaly(7,8). In addition we noted a new finding wherein parental thymocytes and F_1 cells, either spleen (Fig. 1) or LN (Table 4), acted "antergistically." ("Antergy" is a more proper antonym for synergy than is antagonism, and avoids the implication that one cell acts in opposition to the other; a more complex series of interactions is possible.) This new finding has been analyzed in detail in a separate communication(28) and our primary concern here is with its effect on the interpretation of the data. The question we have asked is "does the interaction between parental and host (F_1) cells, which occurs in GVH reactions, confer any

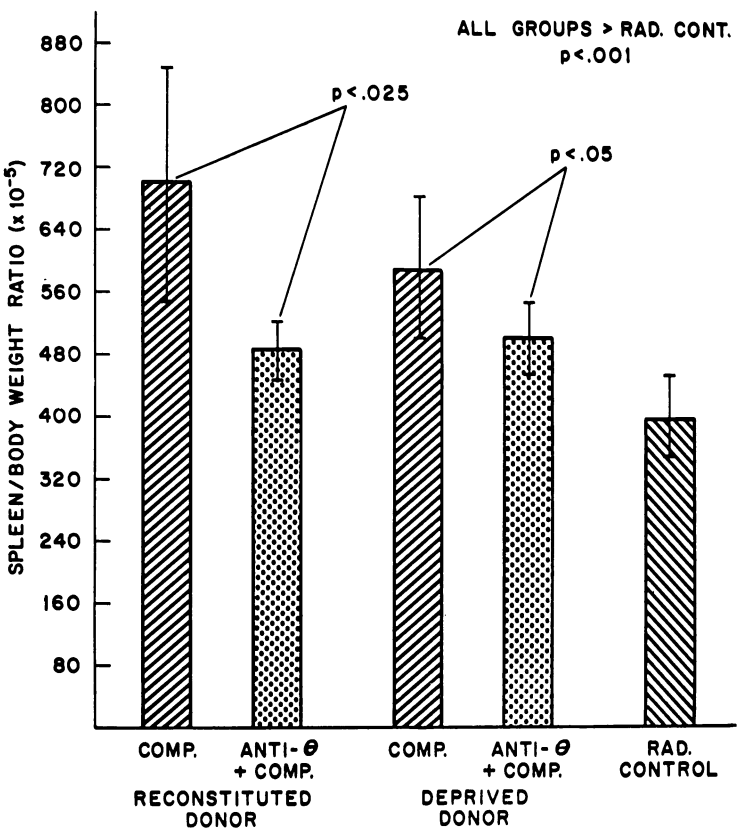

FIG. 2. Spleen indices of sublethally irradiated CDF_1 mice given spleen cells from thymus-deprived C3H mice grafted with a DBA skin graft 2 months previously. Some donors had been reconstituted with 3×10^7 thymocytes. Half of each group of donor cells were treated with anti-theta plus complement and half with complement alone. Each recipient got 2×10^7 (pretreatment count) spleen cells.

autoimmune potential on the host cells?" Since the interactions we reported were synergistic in three experiments and antergistic in two others can we compare interexperiment results? We can if we assume that the increased splenomegaly in any given experiment is due to increased proliferation of host (F_1) cells, either of endogenous or exogenous origin. A number of workers have established that this is the usual case(14–19). We have shown that, under certain conditions the parental thymocytes cooperate with endogenous host (F_1) cells which remain after 350 R and the addition of more host (F_1) cells may prevent the cooperation(28). In these situations then, we think that comparisons of the immune potential of spleen cells which have proliferated excessively (i.e., given greater splenomegaly) can be made with those that have proliferated less, as the increased proliferation is a measurement of the response of the host (F_1) cells.

We found that excess proliferation of host cells was not associated with an increased capacity of the spleen cells to mount an immunologic attack on F_1 hosts. We also found that theta + cells were primarily responsible for the increased GVH activity displayed by spleen cells of both thymus-deprived and thymus-deprived thymocyte-reconstituted parental mice which had been grafted with skin of the reciprocal parent in the F_1 cross. In conjunction, these results suggest that in cellular immunity, in contrast to humoral, most B cells or BMDC do not acquire immune potential in the presence of T cells or that if they do, this results in their becoming theta +.

In addition, we showed that synergistic reactions between parental thymocytes and F_1 cells did not depend on the presence of T cells in the F_1 populations. This also suggests that the increased splenomegaly resulting from interactions between parental and F_1 cells does not depend on the acquisition of any immune potential in the F_1 population.

McNeill has shown that antigenic stimulation results in the production of factor(s) which stimulate hematopoietic stem cells(29–31). The release of such factors during GVH reactions could account for the proliferative reactions of the host cells that we and others(14–19) have described. Elkins has recently put forth a persuasive argument that such is the case(32) and the results we have presented, by failing to find an immunologic component in the proliferative reaction, lend indirect support to that view.

Superficially, it would appear that the basis for the synergistic reactions we have reported, as well as those noted by Hilgard(8), is different from that for the reactions noted by Asofsky, Cantor and Tigelaar(33). The synergy in the production of GVH splenomegaly noted by those authors require that both interacting cells be T cells and also that they both be parental. Their assay is performed in newborn mice however, which might be expected to have sufficient endogenous cells for the parental cells to cooperate with. Our sublethally irradiated mice lack such cells and that is probably why we get synergistic reactions. Nonetheless, one must keep open the possibility that the activation of nonimmunologic F_1 cells by parental T cells is a manifestation or reflection of mechanisms in which they are activating each other.

SUMMARY

The nature of the interaction between parental thymus cells and various host cell populations in the production of graft versus host (GVH) splenomegaly in sublethally irradiated F_1 recipients was investigated. Both synergistic and antagonistic (antergistic) reactions occurred. All F_1 populations investigated (lymph nodes, bone marrow, bone marrow derived spleen, adult spleen and newborn spleen cells) affected the GVH splenomegaly produced by the parental thymocytes. The immunologic consequences of these interactions were investigated.

Retransfer of spleen cells of F_1 mice undergoing GVH reactions gave no evidence that the parental:F_1 interactions which resulted in increased splenomegaly yielded any increased anti-F_1 immune potential of the cells in the spleen. Spleen cells of thymus-deprived C3H mice which had rejected a DBA skin graft had increased GVH potential in CDF1 (C3H $\times$ DBA) recipients, but this was mediated by theta-positive cells, most likely residual T cells. Thus, we suggest that parental:host cell interactions in GVH disease do not convey significant cell mediated autoimmune potential to the host cells and that the proliferation of bone marrow-derived cells as a result of the attack mounted by parental thymocytes is immunologically nonspecific.

REFERENCES

1. Miller, J. F. A. P. and Mitchell, G. F., *Transplant. Rev.* **1,** 3 (1969).
2. Davies, A. J. S., *Transplant. Rev.* **1,** 43 (1969).
3. Claman, H. N. and Chaperon, E. A., *Transplant. Rev.* **1,** 92 (1969).
4. Taylor, R. B., *Transplant. Rev.* **1,** 114 (1969).
5. Cantor, H., Asofsky, R. and Talal, N., *J. Exp. Med.* **131,** 223 (1970).
6. Cantor, H. and Asofsky, R., *J. Exp. Med.* **131,** 235 (1970).
7. Barchilon, J. and Gershon, R. K., *Nature (London)* **227,** 71 (1970).
8. Hilgard, H. R., *J. Exp. Med.* **132,** 317 (1970).
9. Elkins, W. L., *J. Exp. Med.* **123,** 103 (1966).
10. Asherson, G. and Zembala, M., *J. Exp. Med.* **132,** 1 (1970).
11. Eidinger, D. and Ackerman, A., *J. Exp. Med.* **135,** 5 (1971).
12. Lubaroff, D. M. and Waksman, B. H., *J. Exp. Med.* **128,** 1425 (1968).
13. Volkman, A. and Gowans, J. L., *Brit. J. Exp. Pathol.* **46,** 62 (1965).
14. Davies, A. J. S. and Doak, S. M. A., *Nature (London)* **187,** 610 (1960).
15. Howard, J. G., Michie, D. and Simonsen, M., *Brit. J. Exp. Pathol.* **42,** 478 (1961).
16. Fox, M., *Immunology* **5,** 489 (1962).
17. Auerbach, R. and Globerson, A., *Exp. Cell Res.* **42,** 31 (1966).
18. Nakic, B., Kastelan, A., Mikuska, J. and Bunarevic, A., *Immunology* **12,** 615 (1967).
19. Elkins, W. L., *Transplantation* **9,** 3 (1970).
20. Gershon, R. K. and Kondo, K., *Immunology* **18,** 723 (1970).
21. McCullagh, P. J., *J. Exp. Med.* **132,** 916 (1970).
22. Simonsen, M., *Prog. Allergy* **6,** 349 (1962).
23. Hilgard, H. R., *Transplantation* **10,** 5 (1971).
24. Dresser, D. W. and Mitchison, N. A., *Advan. Immunol.* **8,** 129 (1968).

25. Reif, A. E. and Allen, J. M. U., *J. Exp. Med.* **120,** 413 (1964).
26. Billingham, R. E. and Medawar, P. B., *J. Exp. Biol.* **28,** 385 (1951).
27. Raff, M. C., *Nature (London) New Biol.* **229,** 182 (1971).
28. Liebhaber, S. A., Barchilon, J. and Gershon, R. K., *J. Immunol.* In press.
29. McNeill, T. A, *Immunology* **18,** 19 (1970)
30. McNeill, T. A., *Immunology* **18,** 49 (1970).
31. McNeill, T. A., *Immunology* **18,** 61 (1970).
32. Elkins, W. L., *Prog. Allergy* **15,** 70 (1972).
33. Asofsky, R., Cantor, H. and Tigerlaar, R. E. *in Progress in Immunology* (B. Amos, ed.), p. 369. Academic Press, New York, 1971.