Proc. Nat. Acad. Sci. USA
Vol. 68, No. 4, pp. 699–703, April 1971

Radioresistance of the Enhancing Effect of Cells from Carrier-Immunized Mice in an *In Vitro* Primary Immune Response

J. KETTMAN AND R. W. DUTTON

Department of Biology, University of California, San Diego, La Jolla, Calif. 92037

Communicated by S. J. Singer, January 13, 1971

ABSTRACT **The *in vitro* primary response of mouse spleen cell suspensions to 2,4,6-trinitrophenyl(Tnp)-erythrocytes has been studied. The number of anti-Tnp plaque-forming cells that arise after antigenic stimulation *in vitro* is greatly enhanced by prior immunization *in vivo* with the carrier erythrocyte. The enhancement is antigen specific. The priming for an enhanced response can be elicited with very low antigen doses and is often apparent 24 hr after immunization. It is marked from day 3 to day 14. Spleen cells from carrier-primed mice will enhance the anti-Tnp response of normal cells when mixed cultures of the two cell populations are challenged with Tnp-erythrocytes *in vitro*. The carrier-primed cells mediating this enhancing effect are thymus derived.**

The development of the thymus-derived, carrier-specific cell population has been generally assumed to involve the antigenic stimulation of cell proliferation. It was, therefore, somewhat surprising to find that the enhancing effect of the carrier-primed cells, once they had been generated, is not inhibited by x-irradiation.

Observations made with several different experimental models support the hypothesis that two or more cells are required for the immune response. Thus, (*a*) synergistic effects have been observed between bone marrow and thymus cells when injected into irradiated recipients (1, 2), and the depressed response of neonatally thymectomized mice is restored by injection of thymus or thoracic duct cells (3). Similar effects were observed *in vitro* (4, 5). (*b*) Synergistic effects were also seen when certain cells, separated on the basis of physical properties, such as differential attachment to surfaces (6–11) or differential sedimentation in density gradients (12–16), were incubated together *in vitro*. The nature of the helper function of the attached cells in these studies is not yet fully understood, but it would appear to represent the activity of a third cell in addition to the antigen-specific bone marrow and thymus-derived cells described above. (*c*) Prior injection of the carrier antigen enhances the subsequent response to a hapten coupled to the same carrier (17, 18), and synergistic effects are seen in the response to a hapten–carrier conjugate of irradiated recipients that have been injected with two populations of cells, one immunized with the carrier and the other with the hapten (19), or when carrier-primed cells are injected into hapten-primed recipients (20). Similar synergistic effects were observed by Breitner and Miller (21) and by Trowbridge *et al.* (22) in *in vitro* systems.

In many of these experiments, it has been shown that one population supplies the bone-marrow-derived precursor cell while the other population supplies a cell or cells that performs some helper function (2, 5, 11, 14, 19, 23, 24).

Recent experiments have demonstrated some of the interrelationships between these different cooperative effects. Thus, it has been shown that the carrier-primed cells that enhance the hapten response are thymus-derived (25, 26).

We are concerned here with the characterization of the bone marrow–thymus cell cooperation. We have previously reported a primary immune response against the 2,4,6-trinitrophenyl group (Tnp), a haptenic determinant covalently fixed to an erythrocyte carrier (27), when mouse spleen cell suspensions were stimulated *in vitro* (28).

In this paper we show that the primary *in vitro* response to a hapten–carrier complex is enhanced by prior immunization with the carrier, and describe experiments in which we have used this system to investigate further the properties of this helper effect of the thymus-derived cell. The principal findings are concerned with the kinetics of the development of "helper"-cell populations and the demonstration that "helper"-cell activity, once developed, is not inhibited by x-irradiation. A preliminary account of some of these experiments has been published (26), and somewhat similar observations have since been reported by Katz *et al.* (29), who studied the enhancement of a secondary response to a hapten, *in vivo*, in guinea pigs.

MATERIALS AND METHODS

Animals

BDF_1 (C57BL/6 female × DBA/2 male) mice were used throughout unless otherwise indicated and were bred in our own colony. C57BL/6 and DBA/2 were purchased from Jackson Laboratories, Bar Harbor, Me.

Antigens

2,4,6-trinitrophenyl(Tnp)-substituted erythrocytes (RBC) of the sheep, burro, horse, or chicken were prepared as described by Rittenberg and Pratt (30). Heavily substituted erythrocytes were used as immunogen and lightly substituted erythrocytes as antigen in the assay of antibody-forming cells (plaque-forming cells, PFC) (27). 1.2×10^7 mouse spleen cells were cultured in 1 ml of a modified Eagle's medium containing 5% fetal bovine serum. The cultures were assayed 2, 3, 4, and 5 days after the addition of antigen. The direct hemolytic plaque assay of Jerne was used to determine the number of antibody-forming cells. The Tnp response of cultures immunized with hapten conjugated to one carrier was assayed using hapten coupled to a second, noncrossreacting carrier (27). The results are expressed as the number of plaque-form-

Abbreviations: Tnp, trinitrophenyl; RBC, red blood cell; PFC, antibody-forming cells (plaque-forming cells); Dnp, dinitrophenyl.

TABLE 1. *Enhancement of anti-hapten response by prior immunization with carrier erythrocytes*

Expt.	*In vivo* immunization	*In vitro* antigen	Anti-Tnp response* on day 2	3	4	5	Anti-sheep response† on day 2	3	4	5
1	None	Tnp-(sheep RBC)	62	42	65	185	15	95	865	1955
	Sheep		107	118	412	622	3021	9198	6536	2593
	Chicken		35	57	55	79	41	217	582	595
2	None	"	3	1	64	308	2	15	900	2426
	Sheep		10	32	1000	350	335	2410	6200	900
	Chicken		3	4	59	259	1	58	915	1085
3	None	"		9	98	353		32	740	2200
	Sheep			148	2620	1650		4043	36200	8500
	Chicken			29	213	58		109	725	600
4	None	"		35	290	140		122	685	1170
	Sheep			502	2150	64		4561	5750	1260
5	None	"	1	10	170	1083	1	6	747	5666
	Sheep		7	18	1341	2474	114	2290	86138	9102
6‡	None (C57BL/6J)	"			178	238			687	1322
	Sheep				938	1439			16700	67000
	None (DBA/2J)	"			810	862			234	534
	Sheep				3522	3063			8365	5702
	None (BDF_1J)	"			386	261			540	750
	Sheep				1638	1592			11795	20000
7	None	"		76	176	28	5	320	1599	1414
	Sheep			664	2418	583	921	14765	37636	11532
8	None	"	36	44	196	232	17	78	810	3690
	Sheep		44	278	1190	950	3030	15700	60000	50000
9	None	"		84	555	1100		65	440	4800
	Burro			76	530	1080		165	810	4200
10	None	" + (sheep RBC)		12	90	314		71	486	5300
		" + (chicken RBC)		15	179	242		48	850	2250
	Sheep	" + (sheep RBC)		196	1610	2800		5500	36400	11600
		" + (chicken RBC)		86	1350	2960		2845	8600	4300
	Chicken	" + (sheep RBC)		29	213	58		109	723	600
		" + (chicken RBC)		44	165	50		187	456	148

All primed mice were injected (i.v.) with 0.2 ml of 0.5% (v/v) of various RBC suspensions as indicated, 3 days before they were killed. Cell suspensions were cultured under standard conditions and the antigens were added as indicated. The anti-Tnp and anti-(sheep RBC) responses were assayed on days 2, 3, 4, and 5.

* Anti-Tnp PFC/10^6, assayed on Tnp-(burro RBC) with the control result subtracted. Expt. 9 was assayed on Tnp-(horse RBC).

† Anti-(sheep RBC) PFC/10^6.

‡ BDF_1 mice were used in all experiments except Expt. 6.

ing cells per 10^6 recovered cells, unless otherwise indicated. Some cell suspensions were γ-irradiated with a ^{60}Co source.

RESULTS AND DISCUSSION

When spleen cell suspensions from normal mice are incubated with 3×10^5 Tnp-(sheep erythrocytes), there is an exponential rise in the number of anti-Tnp cells. There are generally 100–800 such antibody-forming cells per 10^6 recovered cells by day 4 (27).

The number of PFC is greatly enhanced if the spleen cell donor is immunized with 0.2 ml of a 0.5% v/v suspension of the carrier sheep erythrocytes 3 days before the donor is killed (Table 1). In other experiments (not documented here) it was shown that this antigen dose was more effective than a higher dose (0.2 ml of 10% v/v sheep RBC suspension). In some cases, priming was achieved with antigen doses as low as 0.2 ml of a 0.005% suspension when no increase of PFC could be observed at the time the mice were killed (Falkoff and Kettman, unpublished observations). The enhancing effect is antigen-specific. Thus, immunization with a noncrossreacting carrier, chicken RBC or burro RBC, was without effect (Expts. 1, 2, 3, 9, and 10; Table 1). The simultaneous addition of Tnp-(sheep RBC) plus chicken RBC in chicken RBC-immunized mice gave no enhancement (Expt. 10, Table 1). The enhancing effect was also observed with C57BL/6 and DBA/2 mice (Expt. 6, Table 1).

In the next experiment, we tested whether the Tnp response of spleen cells from normal mice could be enhanced by the presence of cells from a second group of mice injected 3 days previously with the carrier antigen. The spleen cells from the carrier-primed mice were irradiated with 1000 R, which prevented them from giving rise to any anti-Tnp cells. The results illustrated in Fig. 1*a* show that the presence of carrier-primed cells does enhance the anti-Tnp response of spleen cells from normal mice and, furthermore, that this enhancing effect is not abolished by exposure to 1000 R (Fig. 1*b*). The addition of irradiated normal cells was without effect, Fig. 1*c*. The kinetics of the responses are illustrated in Table 2. It can be seen that the enhancing effect was only slightly reduced after exposure to 4000 R. In these experiments, packed spleen

TABLE 2. *Irradiation insensitivity of the enhancing effect of carrier-primed cells: kinetics of the respones*

Cell suspensions*	Anti-Tnp response on day				Anti-sheep response on day			
	2	3	4	5	2	3	4	5
Normal	36	44	196	232	17	78	810	3690
9/10 N + 1/10 SPr	25	128	540	575	145	1430	4500	5320
7/10 N + 3/10 SPr	41	162	1770	985	602	5780	7750	28000
5/10 N + 5/10 SPr	24	212	1660	1520	1069	7900	21800	32000
SPr	44	278	1190	950	3030	15700	60000	50000
9/10 N + 1/10 1K SPr	20	36	200	1240	35	328	1340	2540
7/10 N + 3/10 1K SPr	14	79	775	970	151	1170	3240	2220
5/10 N + 5/10 1K SPr	18	89	1550	780	344	1950	7000	3860
1K SPr	10	13	120	15	1241	3370	4000	3020
9/10 N + 1/10 4K SPr	16	27	335	310	10	117	940	2140
7/10 N + 3/10 4K SPr	30	86	304	555	25	222	1040	1970
5/10 N + 5/10 4K SPr	17	52	560	900	31	280	235	1710
4K SPr	1	2	2	0	693	7	37	7
9/10 N + 1/10 1K N	29	19	155	173	13	38	565	2600
7/10 N + 3/10 1K N	13	20	103	145	13	65	780	1670
5/10 N + 5/10 1K N	8	9	70	145	3	37	940	2720
1K Normal	5	1	1	1	2	1	6	25

Aliquots of a spleen cell suspension from normal mice were incubated alone or diluted in cells from carrier-primed mice. Packed spleen cells were irradiated with 1000 or 4000 R. Primed mice were injected intravenously with 0.2 ml of 0.5% (v/v) sheep RBC 3 days before the mice were killed. Data expressed as PFC/10^6 recovered cells.

* SPr = (sheep RBC)-primed; N = normal; 1K, 4K = given 1000 or 4000 R.

cell suspensions were irradiated and the primed response to the carrier (sheep RBC) was not completely suppressed by 1000 R. In subsequent experiments, cells were irradiated in suspension and the carrier PFC response was reduced to zero, while the enhancing effect remained unaffected.

The antigen specificity of this enhancing effect is demonstrated in Fig. 2. When the *in vitro* immunogen is Tnp-(sheep RBC), priming with sheep RBC, but not with burro RBC, is effective. When the *in vitro* immunogen is Tnp-(burro RBC), priming with burro RBC, but not with sheep RBC, is effective.

The enhancing effect of carrier priming appeared to be a general phenomenon, not restricted to the Tnp response.

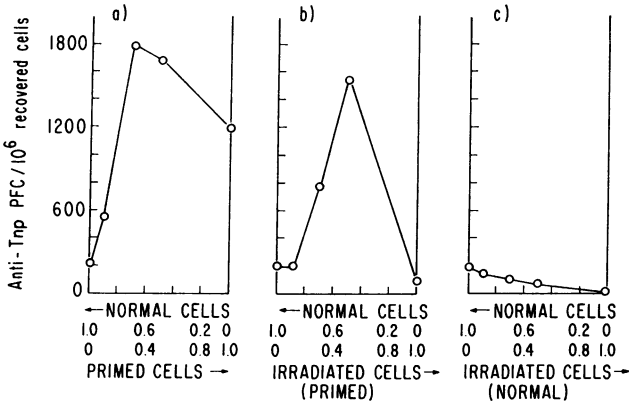

FIG. 1. Enhancement of anti-Tnp Response of spleen cells from normal mice with cells, or irradiated cells, from carrier-primed mice. Carrier-primed mice were injected with 0.2 ml of 0.5% sheep RBC 3 days before they were killed. Packed spleen-cell suspensions from carrier primed or normal mice were irradiated with 1000 R. Aliquots of a pool of spleen cells from normal mice were incubated alone or diluted in carrier primed (*a*), irradiated carrier-primed (*b*), or normal spleen cells (*c*). All cultures contained the same total number of spleen cells and were challenged with Tnp-sheep RBC. Cells making antibody to Tnp were assayed on day 3, 4, and 5 with Tnp-burro RBC. The day 4 response, expressed as PFC/10^6 recovered cells, is illustrated here.

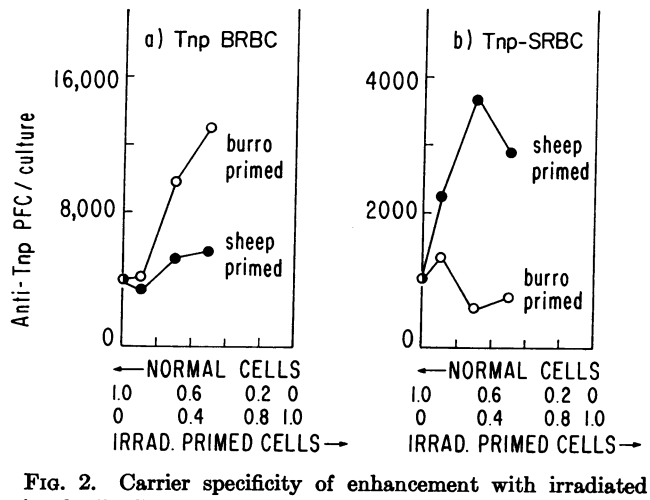

FIG. 2. Carrier specificity of enhancement with irradiated primed cells. Cells from normal mice were diluted in irradiated cell suspensions from either sheep or burro RBC-primed mice injected with 0.2 ml of a 0.5% (v/v) erythrocyte suspension 3 days before they were killed. One set of cultures, (*a*), was challenged *in vitro* with Tnp-burro RBC and the other, (*b*), with Tnp-sheep RBC. In each case, the number of anti-Tnp PFC was measured on days 2, 3, 4, and 5. The results are expressed as PFC/culture, corrected to compensate for the different number of normal cells present. (It can be seen that the anti-Tnp response of normal cells was characteristically higher when challenged with Tnp on burro red cells than on sheep.) For additional details, see text and legend to Fig. 1.

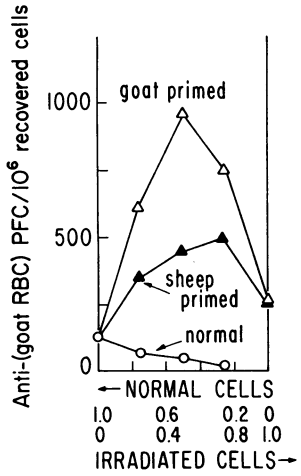

FIG. 3.

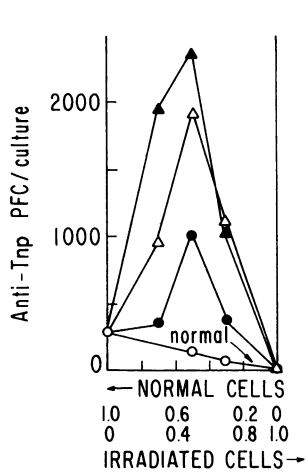

FIG. 4.

FIG. 3. Enhancement of anti-RBC response by carrier priming. Cells from normal mice were diluted in irradiated cell suspensions from either sheep- or goat-primed mice injected with 0.2 ml of 0.5% (v/v) erythrocyte suspensions 3 days before they were killed. Spleen cell cultures were challenged with goat RBC, and assayed against goat RBC, on day 4. The results are expressed as PFC/10^6 recovered cells.

FIG. 4. Time of carrier priming. Samples from a pool of spleen cells from normal mice were diluted in irradiated cell suspensions from mice injected with 0.2 ml of 0.5% (v/v) sheep RBC 1 (●—●) 4 (▲—▲), or 14 (△—△) days before they were killed. All cultures were challenged *in vitro* with Tnp-sheep RBC and assayed for anti-Tnp PFC on days 2, 3, 4, and 5. The PFC/culture that was observed on day 4 is illustrated.

Thus, the addition of irradiated spleen cells from mice primed to sheep or goat RBC enhanced the response of normal cells to goat RBC (Fig. 3).

In the next experiment, we examined how soon the enhancing effect of carrier priming develops. Mice were injected with sheep RBC 1, 2, 4, 6, 8, or 14 days before they were killed and the extent of the anti-Tnp response to Tnp-(sheep RBC) was compared with that of spleen cells from normal mice. In two experiments out of three, the response was already increased by day 1 and the effect was marked from days 3 to 14 (Table 3).

The same effect is shown in experiments where irradiated cells from the mice primed 1, 4, or 14 days before sacrifice were mixed with aliquots from a pool of normal spleen cells. The results of these experiments are illustrated in Fig. 4.

In other experiments we showed that the enhancing effect of irradiated carrier-primed cells could be abolished by prior treatment with antiserum specific for the thymus antigen θ, plus complement (26). The same enhancing effect was shown with thymus-derived cells prepared from the spleens of mice irradiated 7 days previously, and injected with thymus cells and antigen (26). These two pieces of evidence establish the fact that the enhancing effect of carrier-primed spleen cell is mediated by thymus-derived cells. The experiments described are compatible with the hypothesis that a thymus-derived population with specificity for the carrier antigen determinants is required for, or is in some way helpful to, the response to a single identified determinant, such as Tnp. The enhancing activity of carrier-primed cells increases rapidly after antigen injection and can be elicited with very low antigen doses (2×10^4 sheep RBC). It has been suggested that the thymus-derived antigen-reactive cells multiply rapidly after antigenic stimulation (31, 32), and that irradiation of thymus cell suspensions abolished their ability to restore the responsiveness of neonatally thymectomized mice (33). It is, therefore, somewhat surprising that the activity of the carrier-primed cell population, once developed, is not abolished by heavy doses of irradiation. A similar apparent insensitivity to radiation of thymus-derived cells was previously observed in the restoration of spleen cell suspensions from neonatally thymectomized mice (4, 5), and Katz *et al.* (29) have shown that the irradiation of spleen and lymph nodes from guinea pigs immunized with bovine gamma globulin did not inhibit the enhancing effect on the secondary response to Dnp-bovine gamma globulin in recipients immunized against Dnp-ovalbumin. It is interesting to note that in these experiments the radioresistant effect was still seen, even though the antigen challenge was not given until 6 days after the cell transfer.

Several explanations can be offered: (*a*) The helper activity is needed only briefly, at the initiation of the response, and the fact that the cells subsequently divide and die does not prevent their enhancing activity. (*b*) The thymus-derived cells do not divide in the *in vitro* system because of some inadequacy of the culture medium and therefore do not run into any problem from irradiation-induced damage. In the experiment of Katz *et al.* (29), one would have to assume that the cells did not divide in the recipient during the 6-day interval because no antigen was present. (*c*) The development of the carrier-primed thymus-derived population does not involve cell division, but rather maturation of cells already present, or migration of cells from other parts of the body into the spleen.

TABLE 3. *Effect of carrier immunization on anti-hapten response: timing of carrier immunization*

Expt.	Day of immunization	anti-Tnp PFC/10^6 on day 3	anti-Tnp PFC/10^6 on day 4	anti-Tnp PFC/10^6 on day 5	anti-Sheep PFC/10^6 on day 3	anti-Sheep PFC/10^6 on day 4	anti-Sheep PFC/10^6 on day 5
A	0	16	152	35	59	307	600
	−1	71	141	436	1476	2888	3397
	−3	247	613	921	4243	15700	5904
	−7	204	2760	1467	3135	6375	6091
	−3*	66	842	1548	2188	9824	2113
B	0	64	60	...	52	...	...
	−1	127	290	...	940	...	...
	−4	310	2370	...	9700	...	...
	−8	490	1920	...	5900	...	...
	−14	210	680	...	455	...	...
C	0	35	290	140	122	685	1170
	−1	77	490	60	826	2240	1500
	−2	228	1030	70	3652	3600	1390
	−3	502	2150	64	4561	5750	1260

Carrier-primed mice were injected with 0.2 ml of 0.5% (v/v) sheep RBC 1, 2, 3, 4, 7, 8, or 14 days before they were killed, as indicated. Spleen cell suspensions from these and normal mice were incubated *in vitro* with Tnp-sheep RBC, and the anti-Tnp and anti-sheep RBC responses were measured on days 3, 4, and 5. Results are expressed as PFC/10^6 recovered cells.

* The mice received a secondary injection of sheep RBC 3 days before death.

We thank Millie Davenport for her excellent technical assistance and Irving Goldman of the Salk Institute for Biological Studies for the use of radiation facilities.

This work was supported by grants from the National Institutes of Health, USPHS AI-08795-02; the American Cancer Society, E395D; and by an American Cancer Society Faculty Research Award (R.W.D.) PRA-73.

1. Claman, H. N., E. A. Chaperon, and J. L. Triplett, *J. Immunol.*, **97**, 828 (1966).
2. Mitchell, G. F., and J. F. A. P. Miller, *J. Exp. Med.*, **128**, 821 (1968).
3. Mitchell, G. F., and J. F. A. P. Miller, *Proc. Nat. Acad. Sci. USA*, **59**, 296 (1968).
4. Munro, A., and P. Hunter, *Nature*, **225**, 277 (1970).
5. Hirst, J. A., and R. W. Dutton, *Cell. Immunol.*, **1**, 190 (1970).
6. Mosier, D., *Science*, **158**, 1573 (1967).
7. Talmage, D. W., J. Radivich, and H. Hemingsen, *J. Allergy*, **43**, 323 (1969).
8. Pierce, C. W., *J. Exp. Med.*, **130**, 345 (1969).
9. Theis, G. A., and G. F. Thorbecke, *J. Exp. Med.*, **131**, 970 (1970).
10. Hoffmann, M., *Immunology*, **18**, 789 (1970).
11. Hartmann, K., R. W. Dutton, M. M. McCarthy, and R. I. Mishell, *Cell. Immunol.*, **1**, 182 (1970).
12. Haskill, J. S., *Nature*, **216**, 1229 (1967).
13. Haskill, J. S., P. Byrt, and J. Marbrook, *J. Exp. Med.*, **131**, 57 (1970).
14. Shortman, K., E. Diener, P. Russell, and W. D. Armstrong, *J. Exp. Med.*, **131**, 461 (1970).
15. Mishell, R. I., R. W. Dutton, and D. J. Raidt, *Cell. Immunol.*, **1**, 175 (1970).
16. Dutton, R. W., M. M. McCarthy, and R. I. Mishell, and D. J. Raidt, *Cell. Immunol.*, **1**, 196 (1970).
17. Rajewsky, K., V. Schumacher, S. Nase, and N. K. Jerne, *J. Exp. Med.*, **129**, 1131 (1969).
18. Katz, D. H., W. E. Paul, E. A. Goidl, and B. Benacerraf, *J. Exp. Med.*, **132**, 261 (1970).
19. Mitchison, N. A., K. Rajewsky, and R. B. Taylor, *Prague Symp. Develop. Aspects of Antibody Formation and Structure*, ed. J. Sterzl and H. Riha (Academic Press, New York, in press).
20. Paul, W. E., D. H. Katz, E. A. Goidl, and B. Benacerraf, *J. Exp. Med.*, **132**, 283 (1970).
21. Breitner, J. C. S., and J. F. A. P. Miller, *Fed. Proc.*, **29**, 572 (1970).
22. Trowbridge, I. S., E. S. Lennox, and R. R. Porter, *Nature*, **228**, 1087 (1970).
23. Nossal, G. J. V., A. Cunningham, G. F. Mitchell, and J. F. A. P. Miller, *J. Exp. Med.*, **128**, 839 (1968).
24. Roseman, J., *Science*, **165**, 1125 (1969).
25. Raff, M. C., *Nature*, **226**, 1257 (1970).
26. Dutton, R. W., P. Campbell, E. Chan, J. H. Hirst, M. Hoffman, J. Kettman, J. Lesley, M. McCarthy, R. I. Mishell, D. J. Raidt, and D. Vann, *2nd Int. Convocation Immunology*, in press.
27. Kettman, J., and R. W. Dutton, *J. Immunol.*, **104**, 1558 (1970).
28. Mishell, R. I., and R. W. Dutton, *J. Exp. Med.*, **126**, 423 (1967).
29. Katz, D. H., W. E. Paul, E. A. Goidl, and B. Benacerraf, *Science*, **170**, 462 (1970).
30. Rittenberg, M. B., and K. L. Pratt, *Proc. Soc. Exp. Biol. Med.*, **132**, 575 (1969).
31. Miller, J. F. A. P., and G. F. Mitchell, *J. Exp. Med.*, **128**, 801 (1968).
32. Shearer, G. M., and G. Cudkowicz, *J. Exp. Med.*, **130**, 1243 (1969).
33. Miller, J. F. A. P., and G. F. Mitchell, *J. Exp. Med.*, **128**, 801 (1968).