PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 151, 12-15 (1976)

Effects of Masking H-2 and Ir Gene Products on the *In Vitro* Responses of Cells from Immunized and Naive Mice (39133)[1]

MARVIN L. TYAN

Dental Research Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514

Work from several laboratories has shown that antisera raised against the gene products of the major histocompatibility locus of the mouse, guinea pig, and man can react with membrane components of macrophages and/or lymphocytes and without killing them impair their *in vitro* responses to allogeneic cells and other thymus-dependent antigens (1–4). Studies conducted in this laboratory have suggested that antibodies reacting with H-2K, H-2D, or Ir-1 (Ia antigens; 5) gene products, as well as those reacting with the entire H-2 complex, are effective in depressing antibody synthesis *in vitro* (4, 6, 7). However, two recent reports have presented data that conflict with both these observations and each other. Frelinger *et al.* (8) reported that antisera against Ia antigens inhibited both primary (IgM) and secondary (IgG) immune responses to burro red blood cells, but that antisera to H-2K or H-2D had no effect on either response. On the other hand, Pierce *et al.* (9) have stated that antisera to Ia antigens and to H-2D products had no effect on the primary response to sheep red cells (SRBC) or to GAT_{10} (a linear polymer of glutamic acid, alanine, and tyrosine) but that an antiserum that reacted with H-2K and Ia antigens inhibited IgM and IgG primary responses.

A review of data gathered in this laboratory on the effects of specific antisera on primary and secondary responses to SRBC suggested that at least part of the apparent conflict between these and the cited studies might be due to differences in culture conditions, in antisera, and, more importantly, to the period of time responder cells had been exposed to antigen before they were assayed. Presented below are observations showing that direct and indirect antibody responses are impaired by antisera to H-2K, H-2D, and Ia only when the responding cells are obtained from unimmunized mice. It was found that as lymphoid cells reacted to antigen and progressed toward a fully matured antibody response, they first became resistant to inhibition by anti-H-2K and H-2D sera, and somewhat later they lost their vulnerability to agents that reacted with Ia antigens. It is felt that these studies reconcile at least some of the differences mentioned above and add to our understanding of the mechanisms by which these antisera impair the kinetics of the antibody response.

Materials and methods. Mice. Adult male CBA/J, C57BL/6J, and $C3D2F_1$ mice were purchased from Jackson Laboratories, Bar Harbor, Maine. B10.AQR and B10.6R mice were bred in this laboratory; the original breeding pairs were generously donated by Dr. D. C. Shreffler, Department of Human Genetics, University of Michigan School of Medicine. The H-2 haplotypes of these mice are listed in Table I.

Antisera. C57BL/6 anti-CBA/J, $C3D2F_1$ anti-B10.AQR, and B10.6R anti-B10.AQR sera were produced by multiple injections of washed spleen cells as described previously (4). (B10 X LP.RIII) anti-B10.A(5R) serum was obtained from Jackson Laboratories. All antisera and normal sera were heat-inactivated at 56° for 20 min and stored at −20°. The predicted interactions of the antisera with specific responder cells are outlined in Table I.

Aggregated proteins. Human IgG and albumin (Miles Laboratories, Kankakee, Ill.) were heat aggregated at 63° for 20 min (10). Heat aggregated IgG has been shown to inhibit *in vitro* antibody responses (6, 7) and to impair the effector arm of antibody-dependent cell-mediated immunity (10) by combining with the Fc receptor on B cells (11).

[1] This work was supported by USPHS Research Grants HD 07708, DE 02668, and RR 5333.

TABLE 1. PREDICTED INTERACTIONS OF ANTISERA WITH RESPONDER CELLS

Antiserum (titer)[a]	Responder strain (H-2 haplotype; H-2K-Ir-Ss-H-2D)		
	B10.AQR (q-k-d-d)	B10.6R (q-q-q-d)	CBA/J (k-k-k-k)
B10.6R anti-B10.AQR (1:128)	Ir^k	None	Ir^k
$C3D2F_1$ anti-B10.AQR[b] (1:128)	$H\text{-}2K^q$ (17)[c] IgG allotype non-H-2 ?	$H\text{-}2K^q$ (17) IgG allotype non H-2 ?	None
(B10 × LP.RIII) anti-B10.A(5R) (1:128)	$H\text{-}2D^d$ (4,13)	$H\text{-}2D^d$ (4,13)	None
C57BL/6 anti-CBA/J (1:256)	$H\text{-}2K^q$ (11), Ir^k, $H\text{-}2D^d$ (3)	$H\text{-}2K^q$ (11), $H\text{-}2D^d$ (3)	$H\text{-}2^k$
Aggregated IgG	Ir (Ia)[d]	Ir (Ia)	Ir (Ia)

[a] Cytotoxic titer determined by trypan blue exclusion.
[b] This antiserum does not impair the function of strains reacting only with the anti-allotype antibodies (e.g., C57BL/6, ref. 7).
[c] H-2 specificity.
[d] Fc receptor on B cells.

Culture conditions. Primary and secondary responses to SRBC were elicited *in vitro* under conditions described previously (12). Briefly, an underlay of washed SRBC (2%) in 1.5 ml of agar (0.25%) was placed in 35 × 10-mm tissue culture dishes (Falcon Plastics, Oxnard CA) and 4 ml of spleen cell suspension (20×10^6 nucleated cells) were added after the agar had hardened. Sera (1:50) were added, and the cells were cultured at 37° in 5% CO_2 in air for 3 days. At this point the medium and cells were discarded and 1 ml of complement (1:10) with or without rabbit anti-mouse IgG serum (RAMIgG, 1:20) was added to each dish. After 1½ hr at 37° the areas of focal hemolysis (foci) were counted. All cultures were done in quadruplicate and replicate samples were generally within 5% of the mean. The culture medium was Eagle's minimum essential suspension medium augmented with nonessential amino acids, L-glutamine, 10% heat-inactivated fetal calf serum, and penicillin and streptomycin (100 units and 100 μg/ml, respectively).

Plaque-forming cells (PFC). Spleen cells were assayed for direct and indirect PFC by a slide modification of the Jerne technique (12).

Immunization. Mice were injected iv with 0.2 ml of 10% SRBC, 1 to 3 times, at weekly intervals, and they were killed 3 to 16 days after the last injection.

Results. When CBA/J, B10.6R, and B10.AQR mice were immunized with SRBC twice and their spleen cells were cultured with SRBC 1 or 2 weeks after the last injection, the number of direct and indirect foci produced by these cells was not impaired by sera that reacted with H-2K, H-2D, or both, but direct foci were moderately inhibited and indirect foci were greatly decreased by aggregated IgG and by an antiserum that reacted with Ia antigens (Table II). By contrast, in parallel experiments the direct response by spleen cells from unimmunized mice was markedly impaired by antisera to H-2K, H-2D, Ia and by aggregated IgG. NMS and aggregated albumin had little effect on plaque formation. The antisera used in these experiments were effective in inhibiting immune responses only when they contained antibody specificities able to react with H-2K, H-2D, or Ia antigens of the responder cells. As shown previously (4, 7), antisera reacting only with responder cell IgG allotypes were not able to depress these responses.

When similar experiments were ordered with respect to the number of SRBC injections and the time between the last injection and exposure to antigen and antisera *in vitro,* it was found that (i) by 5 days after the initial exposure to SRBC *in vivo*, direct PFC were resistant to inhibition by anti-Ia sera and aggregated IgG, (ii) 3 days after a second challenge, indirect foci were only moderately impaired by anti-H-2 sera, but they were still easily inhibited when Ia antigens were masked, (iii) 7 days after a second injection of SRBC direct foci were decreased by anti-Ia but not anti-H-2K sera

while the indirect response was resistant to both, and (iv) 16 days after a second challenge, both direct and indirect responses were again inhibited by blocking Ia antigens but they were unaffected by an antiserum reacting with H-2K (Table 3).

In two experiments, mice were injected with SRBC three times and 5 days after the last injection they were killed, their spleen cells were incubated with aggregated IgG or albumin for 2 hr, and they were then assayed for direct and indirect PFC by the Jerne technique. It was found that this short-term incubation with aggregated IgG had no effect on the number of direct or indirect PFC found on the slides or on the gross size of the individual plaques (data not shown).

Discussion. The observations presented here show that agents capable of masking certain Ia antigens are able to inhibit the early phases of primary and secondary responses to SRBC *in vitro* but that antisera to H-2K and H-2D gene products are effec-

TABLE 2. EFFECTS ON THE *IN VITRO* RESPONSE TO SRBC OF MASKING Ia, H-2K, AND H-2D ANTIGENS ON SPLEEN CELLS FROM IMMUNIZED AND NAIVE B10.AQR MICE.[a]

Donor	Type of focus		Number of hemolytic foci (% of control)						
			NMS (1:50)	Aggregated Albumin (200 μg/ml)	Aggregated IgG (200 μg/ml)	Anti-Ir[b] (1:50)	Anti-H-2K[c] (1:50)	Anti-H-2D[d] (1:50)	Anti-H-2K and H-2D[e] (1:50)
Immunized	Direct	132	104	125	61	57	107	124	150
		(100)	(79)	(95)	(46)[g]	(43)[g]	(81)	(95)	(114)
	Indirect[f]	91	93	88	20	34	100	111	96
		(100)	(102)	(97)	(22)[g]	(37)[g]	(110)	(122)	(106)
Naive	Direct	57	63	56	17	19	18	20	9
		(100)	(110)	(98)	(30)[g]	(34)[g]	(32)[g]	(35)[g]	(15)[g]

[a] The results are representative of data obtained in six other experiments using spleen cells from B10.AQR, B10.6R, and CBA/J mice. The immunized mice were used 1 to 2 weeks after the last injection with SRBC.
[b] B10.6R anti-B10.AQR. No effect on B10.6R cells.
[c] $C3D2F_1$ anti-B10.AQR. No effect on CBA/J cells.
[d] (B10 × LP.RIII) anti-B10.A(5R). No effect on CBA/J cells.
[e] C57BL/6 anti-CBA/J. Data is normalized from an experiment with B10.6R cells. This serum impaired responses from immunized B10.AQR and CBA/J mice because of anti-Ir^k specificities.
[f] Number of foci on plates developed with RAMIgG minus number on plates with complement only.
[g] $P < 0.01$; Student's t test.

TABLE 3. EFFECTS ON DIRECT AND INDIRECT HEMOLYTIC FOCI OF MASKING Ia OR H-2K AT VARIOUS TIMES AFTER IMMUNIZATION WITH SRBC.[a]

Experiment	Number of injections	Last injection (days)	Percentage of hemolytic foci in cultures with NMS			
			Anti-H-2K (1:50)[b]		Anti-Ia or aggregated IgG (1:50)	
			Direct	Indirect	Direct	Indirect
505	0	0	32[c]		26[c]	
518	1	5			77	None
515	2	3	63	57[d]	94	16[c]
505	2	4	96	110	75	43[d]
491	2	6	83	67	30[c]	56
407	2	7	95	86	30[c]	110
402	3	7	110	120	35[c]	120
392	2	16	85	103	26[c]	18[c]

[a] B10.AQR and B10.6R mice were donors of spleen cells. Ia was masked in B10.AQR mice with B10.6R anti-AQR serum, and aggregated IgG (200 μg/ml) was used to block the Fc receptor on B10.6R spleen cells.
[b] $C3D2F_1$ anti-B10.AQR serum.
[c] $P < 0.01$.
[d] $P < 0.05$.

tive only during a primary response. Cells actively synthesizing antibody were not affected significantly by the actions of any of these agents.

Previous work (4, 7) has demonstrated that *in vitro* responses to SRBC may be inhibited by antisera to Ia or H-2 specificities added up to 24 hr after responding cells have been exposed to antigen. The present studies have shown that primary and secondary antibody responses remain vulnerable to the actions of antisera to Ia for a period after becoming resistant to anti-H-2K serum; further, these responses again became susceptible to inhibition by aggregated IgG or anti-Ia sera about 1 week after *in vivo* stimulation with SRBC. Taken together the evidence suggests that antisera to H-2 specificities and Ia antigens act at different stages of the inductive process. Antisera to H-2K and H-2D seem to affect an early nonrecurring phase, perhaps antigen processing or macrophage–lymphocyte interactions (4, 9). The association of the inhibitory properties of anti-Ia sera and of aggregated IgG with their ability to block the Fc receptor on B cells suggests that at least part of their effect on the immune process may be made manifest by masking this receptor site, thus impeding B cell function and/or B cell–T cell cooperation (4, 7, 10).

Frelinger *et al.* (8) reported that when mouse spleen cells were tested 3 weeks after exposure to antigen *in vivo*, IgM and IgG responses were impaired by antisera that reacted with Ia but not H-2K or H-2D antigens. The results obtained in the present studies are in agreement with this, but they also suggest that IgG PFC would have been decreased by antisera to H-2 specificities if the cells had been placed in culture 1 or 2 days after challenge. On the other hand, in contrast to the observations reported here, they found that the primary response to burro RBC was not inhibited by antisera to H-2K or H-2D. Although not certain, it is possible that these differences are due to variations in the culture systems used. Frelinger *et al.* assayed antibody production by viable cells at the end of a 4-day period, whereas the culture system used in these experiments measured the cumulative antibody production of cell clusters during a 3-day period. In view of data (8) indicating that the inhibitory activity of anti-Ia sera may be detected in 4-day but not 5-day-old cultures, it is possible that the inhibitory effects of antisera to H-2 specificities may be manifest after 3 but not 4 days *in vitro*. That is, these sera may retard rather than completely interrupt the *in vitro* response to SRBC.

Finally, the differences between the present results and those reported by Pierce *et al.* (9), who observed no effects of specific antisera to Ia or to H-2D on primary IgM responses may be due to the use of a weak antiserum in the former case (as suggested in Ref. 8) and to the 5-day culture period used in their experiments.

Conclusions. Mouse spleen cells from immunized and naive animals were cultured for 3 days with SRBC and antisera to H-2K, H-2D, Ia, or aggregated IgG. Direct and indirect antibody responses were impaired by each of these agents only when the responding cells had been obtained from unimmunized mice. It was found that as lymphoid cells reacted to the antigen and progressed toward a fully matured antibody response, they first became resistant to inhibition by anti-H-2K and H-2D sera and somewhat later they lost their vulnerability to agents that reacted with Ia antigens.

1. Ceppellini, R., Miggiano, M., Curtoni, E. S., and Pellegrino, M., Transplant. Proc. **3,** 63 (1971).
2. Buckley, R. G., Schiff, R. I., and Amos, D. B., J. Immunol. **108,** 34 (1972).
3. Shevach, E. M., Paul, W. E., and Green, I., J. Exp. Med. **136,** 1207 (1972).
4. Tyan, M. L., Cell. Immunol. **10,** 450 (1974).
5. David, C. S., and Schreffler, D. C., Transplantation **18,** 313 (1974).
6. Tyan, M. L., Fed. Proc. **6,** 999 (1975).
7. Tyan, M. L., Proc. Soc. Exp. Biol. Med., **150,** 847 (1975).
8. Frelinger, J. A., Niederhuber, J. E., and Shreffler, D. C., Science **188,** 268 (1975).
9. Pierce, C. W., Kapp, J. A., Solliday, S. M., Dorf, M. E., and Bennacerraf, B., J. Exp. Med. **140,** 921 (1974).
10. Dickler, H. B., and Sachs, D. H., J. Exp. Med. **140,** 779 (1974).
11. Basten, A., Miller, J. F. A. P., Sprent, J., and Pye, J., J. Exp. Med. **135,** 610 (1972).
12. Tyan, M. L., Ness, D. B., and Gibbs, P. R., J. Immunol. **110,** 1170 (1973).

Received June 30, 1975. P.S.E.B.M., 1976, Vol. 51.