REGULATION OF THE IMMUNE SYSTEM BY SYNTHETIC POLYNUCLEOTIDES

V. Effect on Cell-Associated Immunoglobulin Receptors and Immunological Memory*

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While the presence of immunoglobulin receptor molecules on immunocompetent cells has been detected by a variety of procedures (1), much remains to be learned as to the dynamics of their synthesis and function. Recently the adjuvant action on the immune response of complexed synthetic polynucleotides has been described (2, 3) and characterized (4). The cellular target for this membrane-active adjuvant may be multiple; thus both the macrophage (5) and thymus-influenced lymphocyte (6) appeared to be stimulated by complexes of polyadenylic–polyuridylic acid (poly A:U).[1] In view of this potent action on cells engaged in antibody synthesis, the effect of synthetic polynucleotides on synthesis of cell-associated antibody was studied. The data tabulated herein document that poly A:U alone, without antigen, stimulated the appearance of rosette-forming cells (RFC) in a previously quiescent population of memory cells. This occurred immediately in vitro and after a few hours in vivo. Continued incubation in vitro of spleen cells after exposure to poly A:U resulted in

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[1] *Abbreviations used in this paper*; BME, Eagle's basal medium; bmeS, supernatant from nonstimulated cultures; BSA, bovine serum albumin; ECDI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl; FUDR, 5-fluoro-2-deoxyuridine; GAMIgM, goat anti-mouse IGM; GAM7SGG, goat anti-mouse 7S γ-globulin; HGG, human γ-globulin; hggS, supernatant from HGG-stimulated cultures; NRS, normal rabbit serum; p(a:u)S_{bsa}, supernatant from poly A:U–stimulated cultures of BSA-primed cells; p(a:u)S_{hgg}, supernatant from poly A:U–stimulated cultures of HGG-primed cells; PBS, phosphate-buffered saline; poly A:U, polyadenylic–polyuridylic acid complex; RFC, rosette-forming cells; SRBC, sheep red blood cells.

the appearance of an immunoglobulin G receptor in the culture fluids which was associated with a loss of secondary responsiveness to antigen by such cells. An essential role for this immunoglobulin G receptor in induction of the secondary response was implied by the finding that the reactivity of such unresponsive cells could be restored by addition of supernatant fluids containing the poly A:U–released IgG.

Materials and Methods

Immunization.—Human γ-globulin (HGG), fraction II, B grade (lots 31 and 33) was obtained from Miles Laboratories, Inc., Kankakee, Ill. Bovine serum albumin (BSA), fraction V, lot 7644, was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. 6-wk old BALB/Aj mice were inoculated intravenously with 200 μg of HGG suspended in 0.15 M NaCl. Studies of secondary responsiveness were initiated 6–8 wk later.

Antisera.—Specific goat anti-mouse 7S γ-globulin (GAM7SGG) and goat anti-mouse IgM (GAMIgM) sera were obtained from Meloy Laboratories, Springfield, Va.

Drugs.—Puromycin dihydrochloride, lot No. 6631, was obtained from Nutritional Biochemicals Corp. and 5-fluoro-2-deoxyuridine (FUDR), lot No. 002106, from Hoffman-La-Roche, Inc., Nutley, N.J. Both drugs were suspended in 0.15 M NaCl. Puromycin was incorporated into tissue culture medium to a final concentration of 10 μM (7). FUDR was suspended to a concentration of 8 mg/ml and injected intraperitoneally in a 0.5 ml vol (4 mg/mouse).

Homoribopolynucleotides.—Polyadenylic acid, potassium salt (lots 110748, 11-57-301, 11-65-301, and 11-66-301), and polyuridylic acid, ammonium salt (lots 411754, 11-60-308, 11-71A-301, and 11-75-308), were purchased from Miles Laboratories, Inc., Elkhart, Ind. Each polymer was stored at −20°C in a concentration of 3 mg/ml in 0.15 M NaCl. Polymers were complexed in vitro before use by mixing equal volumes of the two stock solutions for 5 min at room temperature. The polymer was diluted to desired concentration in 0.15 M NaCl and administered intravenously in a volume of 0.3 ml.

Cell Suspensions.—Mice were killed by cervical dislocation and the spleens removed and dispersed in 10 ml of phosphate-buffered saline (PBS), pH 7.2 (0.073 M NaCl, 0.018 M KH_2PO_4, 0.057 M Na_2HPO_4) in a TenBroeck tissue homogenizer. The cell suspensions were washed three times in PBS, pH 7.2. After the second wash, red blood cells were lysed by suspending the packed cells in 5 vol of 0.03 M NaCl, followed in15 sec by the addition of 10–20 vol of PBS,pH 7.2. Splenic histiocytes or macrophages were reduced in number using the technique of Mosier (8). Briefly, the spleen cells were suspended in culture medium to a concentration of 10^8 cells/ml and cultured on glass at 37°C for 30 min without shaking. The culture fluid and nonadherent cells were then transferred to a second flask and cultured for another 30 min period. After three serial cultures, the nonadherent cell suspension consisted of less than 3% macrophages as determined by a differential count of a Wright's-stained smear. Viability of all final cell suspensions was determined by trypan blue exclusion. Dye uptake was determined 2 min after addition of 0.2 ml of trypan blue (0.1% in PBS, pH 7.2) to 1 ml of the cell suspension. Those cell suspensions having a viability of less than 85% were discarded. The viability of spleen cell suspensions used ranged from 93–98%, that of cell suspensions depleted of macrophages ranged from 87–91%.

Spleen Cell Cultures.—Cells were cultured in Eagle's basal medium (BME) (Hyland, Los Angeles, Calif.) in Hanks' balanced salt solution supplemented with 0.2 mM glutamine (Grand Island Biological Co., Grand Island, N.Y.) and 0.1% methylcellulose (15 centipoise, Fisher Scientific Co., Fairlawn, N.Y.). The cells were suspended in medium to a concentration of $1–2 \times 10^7$ cells/ml and dispensed in 8–10-ml aliquots to 50-ml screw-capped Erlenmeyer flasks. Cell suspensions were gassed with a mixture of 95% air–5% CO_2 and cultured at 37°C on a shaking water bath (60 complete cycles/min).

Passive Rosette Assay.—1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (ECDI) was obtained from the Ott Chemical Co., Muskegon, Mich. (lot 1300). The ECDI (100 mg/ml) and the HGG (15 mg/ml) were suspended in PBS, pH 7.2. Sheep red blood cells (SRBC) (Colorado Serum Co., Denver, Colo.) were washed three times in PBS, pH 7.2, and resuspended to 50%. The SRBC were sensitized with antigen by adding 3 ml of the appropriate protein suspension and 0.5 ml of the appropriate ECDI solution to 0.1 ml of 50% SRBC. The mixture was incubated for 1 hr at 4°C with gentle agitation, centrifuged at 400 *g* for 10 min, washed twice in 1% normal rabbit serum (NRS), and resuspended to 5–10% in 1% NRS (31).

Washed monodisperse suspensions of spleen cells, prepared as described above, were suspended in 1% NRS to a concentration of 5–10 $\times$ 10^6 cells/ml. 1-ml aliquots of the cells were added to serum tubes. 0.1 ml of the HGG-treated SRBC was added to each tube, and the tubes were incubated at a 45° angle for 1 hr at room temperature, then overnight at 4°C, When under test, poly A:U was added directly to the cells in the assay tubes to a final concentration of 300 μg/ml at the beginning of the incubation at room temperature. It was found that incubation at 37°C rather than room temperature caused a large increase in nonspecific clumping of the cells. After incubation, the cells were resuspended by gentle inversion. A sample was placed in a hemacytometer and the number of rosettes in the entire chamber counted. The concentration of spleen cells was determined by diluting the cells 1:50 in 2% acetic acid to remove the SRBC and counting the nucleated white blood cells in a hemacytometer.

Serum Antibody Determinations.—Mice were anesthetized with ether and the axillary vessels severed. 0.5–0.7 ml of blood was obtained from each of three to five mice in the group, pooled, and the serum removed and stored at −20°C until use. The antibody content of the serum was determined by the passive hemagglutination technique of Boyden (9). All serum samples collected during a complete experiment were titered at the same time under identical conditions.

Irradiation.—Mice were subjected to whole body irradiation, either sublethal (450 R) or lethal (800 R), at a distance of 100 cm from a ^{60}CO source delivering 67 R/min.

Considerations in Sublethal Irradiation Experiments.—The per cent secondary responsiveness was based on the highest secondary potential obtained in units of RFC per spleen in nonirradiated, primed mice after a standard challenge dose with antigen. The secondary (2°) potential was defined as the number of RFC per spleen obtained after secondary challenge in excess of the number of RFC per spleen obtained by injection of the same antigen dose into nonprimed mice (primary [1°] potential).

$$\text{Per cent } 2^\circ \text{ potential} = \frac{2^\circ \text{ response} - 1^\circ \text{ potential}}{\text{maximum } 2^\circ - 1^\circ \text{ potential}} \times 100.$$

Similarly, the per cent recovery of poly A:U–induced RFC was based on the maximum number of RFC per spleen obtained after injection of poly A:U into nonirradiated, primed mice [max p(A:U):rfc$_2$]. The per cent responsiveness of primed mice to poly A:U was defined as the number of RFC per spleen obtained after injection of poly A:U into primed mice in excess of the number of RFC per spleen obtained by injection of the same quantity of poly A:U into nonprimed mice [p(A:U):rfc$_1$].

$$\text{Per cent p(A:U) responsiveness} = \frac{\text{p(A:U):rfc}_2 - \text{p(A:U):rfc}_1}{\text{max p(A:U):rfc}_2 - \text{p(A:U):rfc}_1} \times 100.$$

In both cases, the recovery of irradiated, primed animals was controlled for primary responsiveness of similarly irradiated, nonprimed animals.

RESULTS

Effect of Poly A:U on Primed Spleen Cells In Vitro.—Spleens from mice given a single injection of 100 μg of HGG alone 6 wk previously contained few RFC.

However, when poly A:U was added to the assay medium a 4-fold increase in RFC was observed repeatedly, together with a slight effect on normal spleen cells (Table I). Due to the rapidity of the reaction and the minimal rosette formation in the absence of poly A:U, it was hypothesized that poly A:U might

TABLE I

Expression of Antibody in Cultures of Primed Spleen Cells Exposed to Poly A:U

Spleen cells	RFC/10^6 cells
Normal	100 ± 100
Normal + poly A:U	310 ± 123
Primed	450 ± 148
Primed + poly A:U	2120 ± 430

Primed spleen cells were obtained from mice injected with 100 μg of HGG 6 wk previously. Poly A:U was added directly to the assay system to a final concentration of 300 μg/ml. RFC values represent the arithmetic mean of six assays; ± values represent the 95% confidence limits.

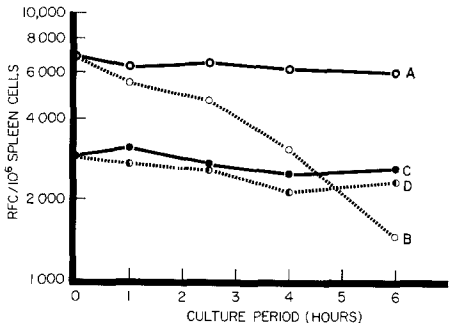

FIG. 1. Effect of puromycin on poly A:U-induced rosette formation. Primed spleen cells obtained from mice injected with 200 μg of HGG 6 wk previously were cultured for 0–6 hr in BME in the presence or absence of 10 μM puromycin. After culture, the cells were washed three times and assayed in the presence of 150 μg/ml of poly A:U excepting for the control cells which were assayed in the absence of poly A:U. Viability of all cell suspensions after culture was >80%. *Group A:* Control spleen cells from primed mice 3 days after secondary challenge with 500 μg of HGG intravenously; cultured in BME alone; assayed in PBS, pH 7.2. *Group B:* As in Group A excepting for culture in 10 μM puromycin; assayed in PBS, pH 7.2. *Group C:* Primed spleen cells cultured in BME alone; assayed in 150 μg/ml of poly A:U. *Group D:* As in Group C excepting for culture in 10 μM puromycin; assayed in 150 μg/ml of poly A:U.

be causing previously synthesized cell-associated antibody to be brought from the interior to the surface membrane of the cell, referred to as "expression" of antibody. To test this, spleen cells from primed mice were cultured without poly A:U for 0–6 hr in the presence or absence of 10 μM puromycin. After culture, the cells were washed and assayed in the presence of poly A:U. As may be seen in Fig. 1, puromycin did not reduce significantly the ability of poly A:U to

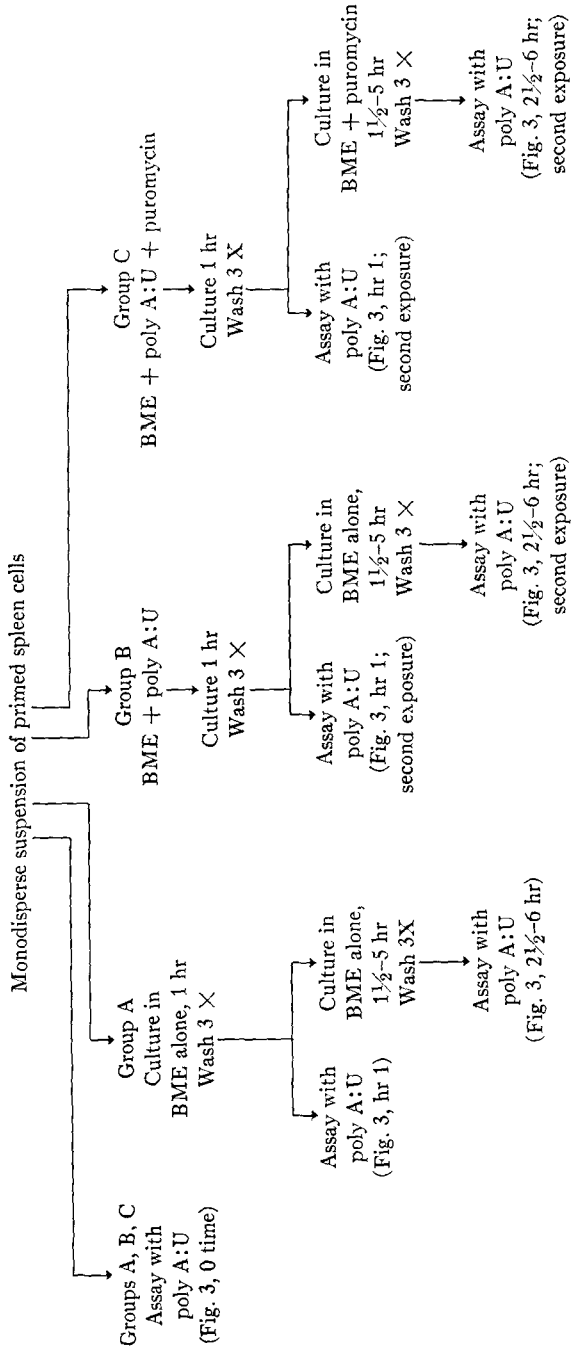

FIG. 2. Protocol for studies on depletion and synthesis of cell-associated antibody.

induce rosette formation. The effectiveness of the puromycin used was documented when antibody synthesis was inhibited in similar control cultures of spleen cells obtained from mice 3 days after secondary challenge of HGG in vivo.

If poly A:U caused the expression of preformed cell-associated antibody as Fig. 1 indicated, the cells might be expected to be less sensitive to a second exposure to poly A:U (i.e., the retained antibody could be depleted by the first contact with poly A:U and removed during the washing of the cells). On the other

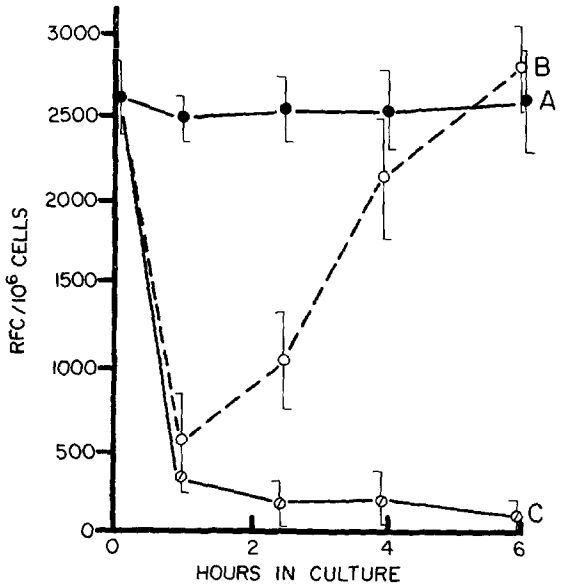

FIG. 3. Effect of poly A:U on culture of primed spleen cells. Primed spleen cells were cultured in BME (*line A*), BME + 300 μg/ml of poly A:U (*line B*), or BME + 300 μg/ml of poly A:U + 10 μM puromycin (*line C*), corresponding to Groups A, B, and C in Fig. 2. Excess poly A:U was removed from the culture fluid by washing the cells after 1 hr of culture and culture continued to 6 hr. All systems were assayed in the presence of 300 μg/ml of poly A:U. Each point represents the arithmetic mean of six cultures. The vertical lines indicate the 95% confidence limits.

hand, the initial loss of antibody through expression to the cell membrane might trigger antibody synthesis on extended culture, an event which should be puromycin sensitive. To investigate this possibility, spleen cells from primed mice were cultured for 1 hr in the presence or absence of poly A:U (first exposure) and/or puromycin, washed and recultured for 0–5 hr in the culture medium with or without addition of puromycin. After the culture period, the cells from all three groups were washed and assayed in the presence of poly A:U (second exposure). The experimental design is shown in Fig. 2, and the results are presented in Fig. 3. The immediate increase in RFC after exposure of all three

groups to poly A:U (0 time) is evident. As may be seen in lines *B* and *C*, Fig. 3, after 1 hr of culture in poly A:U, the cells were no longer sensitive to the second exposure to poly A:U in the assay medium, indicating that cell-associated antibody had been expressed by the first exposure to poly A:U and removed by the wash. However, after removal of excess poly A:U by washing and on further culture, the number of RFC in the cell suspensions increased (line *B*, Fig. 3)

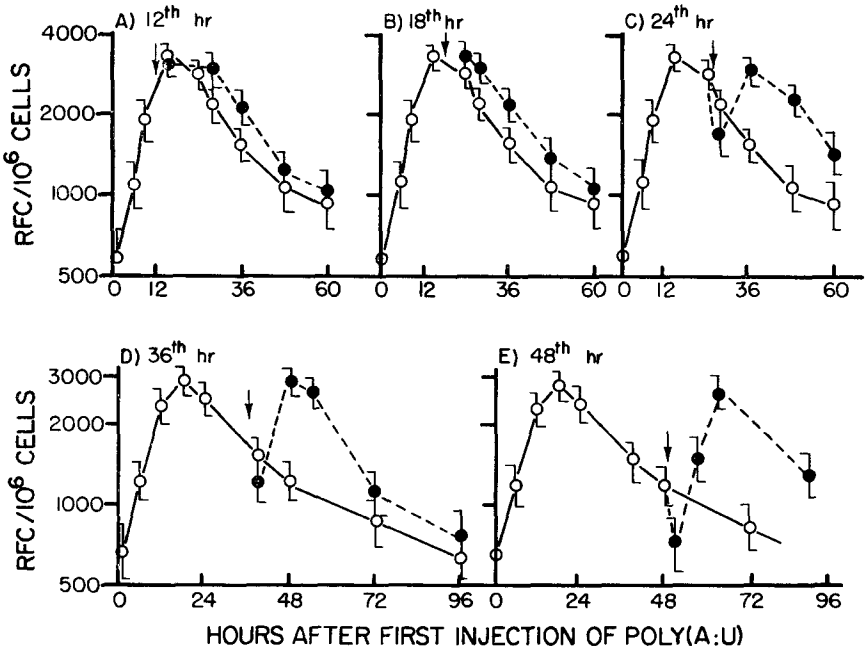

FIG. 4. Effect on RFC of a second injection of poly A:U given at varying intervals after the first injection. Primed mice were injected intravenously with 300 μg of poly A:U (○—○). 12–48 hr later, the mice were injected a second time with either 300 μg of poly A:U (●- -●) or 0.3 ml of saline (○—○). Each point represents the arithmetic mean of three mice. Vertical lines indicate the 95% confidence limits. Arrows (↓) indicate time of second injection of poly A:U.

and this increase was inhibited by puromycin (line *C*, Fig. 3). Repeated experiments revealed a similar pattern of response but with some variance in the exact timing of the events.

The possibility existed that this puromycin-sensitive phase might have been due to the activation of splenic macrophages by poly A:U (5), causing the release of retained antigen and a subsequent specifically induced antibody response. To investigate this possibility, primed spleen cell suspensions of varying glass-adherent cell content were prepared and cultured for 6 hr in the presence or absence of poly A:U before assay. Depletion of the macrophage content

from 10 to 2% did not reduce the intensity of the response to poly A:U (2190 ± 440 vs. 2720 ± 780 RFC/10^6 cells, respectively).

Effect of Poly A:U on Primed Spleen Cells In Vivo.—The nonspecific stimulation by poly A:U of rosette formation by primed spleen cells in vitro was also observed in vivo (10). Increases in splenic RFC were detectable 6 hr after intravenous injection of 300 μg of poly A:U alone, without antigen, into mice immunized 6 wk previously with HGG (Fig. 4). The response peaked 18 hr after injection and had dissipated completely by the 3rd day.

To determine whether the animals' responsiveness to poly A:U was altered by previous exposure to poly A:U, primed mice were treated with two injections

TABLE II

*Effect of FUDR on Poly A:U-Induced RFC Response In Vivo**

Products‡ injected	Time of FUDR injection§	RFC/10^6 cells‖ Hours after poly A:U injection		
		6	21	36
	hr			
None	—	680 ± 168	590 ± 161	750 ± 167
Poly A:U	—	1220 ± 212	2630 ± 326	1460 ± 254
FUDR + poly A:U	−6	1090 ± 216	2470 ± 350	1380 ± 240
	with	1310 ± 195	2480 ± 370	1210 ± 210
	+6		2810 ± 419	1530 ± 266

* 4 mg of FUDR intraperitoneally 18 hr after intravenous injection of 500 μg of HGG into normal mice reduced the primary response from 4820 ± 620 to 1100 ± 390 RFC/10^6 cells (day 8). Normal background level of untreated mice was 540 ± 145 RFC/10^6 cells.

‡ Dosage: poly A:U, 600 μg intravenously; FUDR, 4 mg intraperitoneally.

§ Relative to poly A:U injection at 0 hr.

‖ Numerical values represent arithmetic mean of three mice; ± values represent the 95% confidence limits.

of 300 μg of poly A:U intravenously, spaced 6–72 hr apart. A second injection of poly A:U before the peak of the response to the first injection (either 6 or 12 hr after the first injection) did not significantly alter the response (Fig. 4 *A*) while administration of the second dose of poly A:U at the time of the peak response to the first injection (18th hr) resulted in a slight prolongation of the response. Injection of the second dose of poly A:U after the time of peak response to the first injection (24th hr or later) simply started the response anew without changing its intensity (Fig. 4 *C*, *D*, *E*).

The failure of the first exposure to poly A:U to increase the RFC induced by a subsequent injection of poly A:U indicated that the response did not involve cell division. This was confirmed by the observation that the rise in splenic RFC induced by poly A:U was not significantly depressed by injection of 4 mg

of FUDR intraperitoneally 6 hr before, simultaneously with, or 6 hr after injection of 600 μg of poly A:U intravenously (Table II).

To determine whether a 7S or 19S immunoglobulin was involved in the poly A:U–induced rosette formation, attempts were made to inhibit rosette formation by pretreating the spleen cells with either goat anti-mouse 7S γ-globulin (GAM7SGG) or goat anti-mouse IgM (GAMIgM) sera. Spleens were removed from primed mice 18 hr after intravenous injection of 600 μg of poly A:U. The cells were incubated at 37°C for 45 min in the presence of either GAM7SGG, GAMIgM, or NRS, sedimented to eliminate the excess anti-globulin from the supernate, resuspended in 1% NRS, and assayed for rosette formation. No

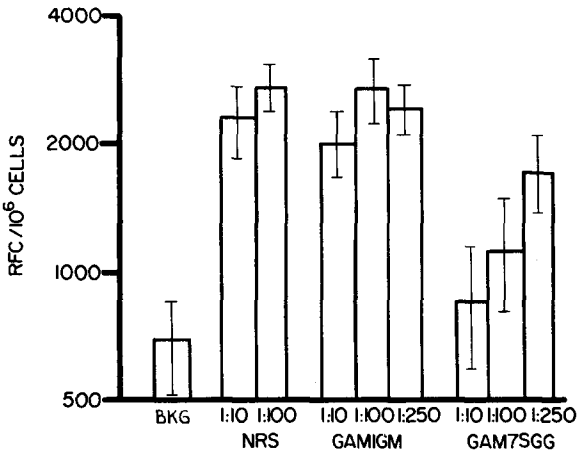

FIG. 5. Effect of anti-gamma globulin sera on rosette formation by poly A:U–stimulated spleen cells. Primed mice were injected intravenously with 600 μg of poly A:U. Spleens were removed and pooled 18 hr later. Cells were incubated 45 min at 37°C with either NRS, GAMIgM, or GAM7SGG, sedimented, resuspended in 1:100 NRS, and assayed for rosette formation. BKG = background RFC of noninjected mice.

significant reduction in RFC was observed in the cell suspensions exposed to GAMIgM. However, there was a >50% reduction in RFC in those cell suspensions exposed to GAM7SGG serum (Fig. 5), indicating an IgG receptor existed on these cells.

Correlation of Poly A:U–Induced RFC with Immunological Memory Cells.—An important question to consider was whether the cells which could be induced to synthesize antibody by poly A:U were involved in secondary responsiveness or were simply quiescent antibody-forming cells remaining from the previous primary response. This was investigated by immunizing mice with either 1, 10, 100, or 1000 μg of HGG intravenously and comparing: (*a*) the peak number of RFC responding to the primary injection of antigen (day 9 after injection); (*b*) the number of cells forming rosettes on incubation with poly A:U in vitro 6

wk after priming; and (*c*) the peak number of cells responding to a challenge dose of 250 μg of HGG (i.e., the secondary response). The peak number of cells responding in the primary response was found to increase as the antigen dose was increased (Fig. 6). In contrast, the number of cells responsive to poly A:U in vitro paralleled the intensity of the secondary antibody response; both plateaued as the priming dose of antigen increased above 100 μg (Fig. 6).

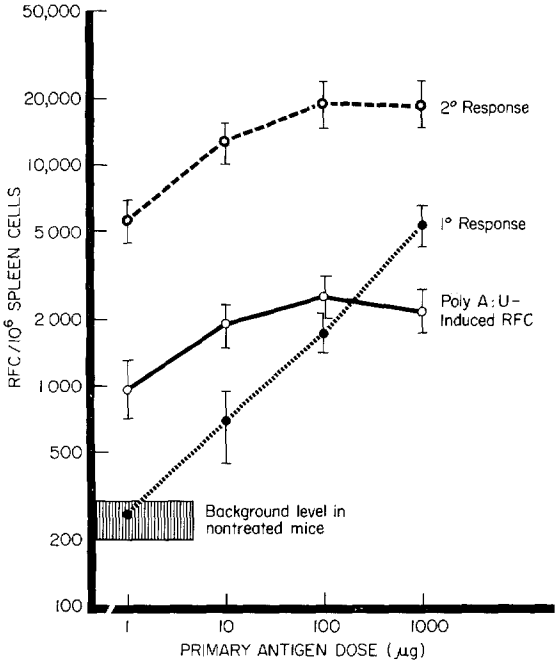

FIG. 6. Relation of poly A:U-induced RFC to primary and secondary antibody-forming potentials. Mice were immunized with 1, 10, 100, or 1000 μg of HGG intravenously. The peak primary (1°) response (●– –●) was measured 9 days after antigen injection; the peak secondary (2°) response (○– –○) was measured 7 days after a second injection of 250 μg of HGG 6 wk after the primary; the number of RFC induced in response to poly A:U was measured by assaying the spleen cells in the presence of 150 μg/ml of poly A:U 6 wk after the primary antigen injection (○—○). Each point represents the arithmetic mean of three mice. Vertical lines indicate the 95% confidence limits.

A second approach to the problem of the functional nature of the cells responsive to poly A:U was based on the observations of Nettesheim and Williams (11) that secondary responsiveness is recovered after low-dose irradiation if the interval between priming and irradiation is short. Thus, mice were irradiated (450 R whole body) 2 wk after priming with 100 μg of HGG. At varying intervals from 1–30 days after irradiation, their spleens were assayed either 24 hr after injection of 300 μg of poly A:U intravenously or 8 days (peak of secondary response) after intravenous challenge with 200 μg of HGG and

the two values compared. As may be seen in Fig. 7, the recovery of cells responsive to poly A:U coincided with the recovery of the secondary antibody-forming potential with respect to time and degree in both irradiated and nonirradiated mice.

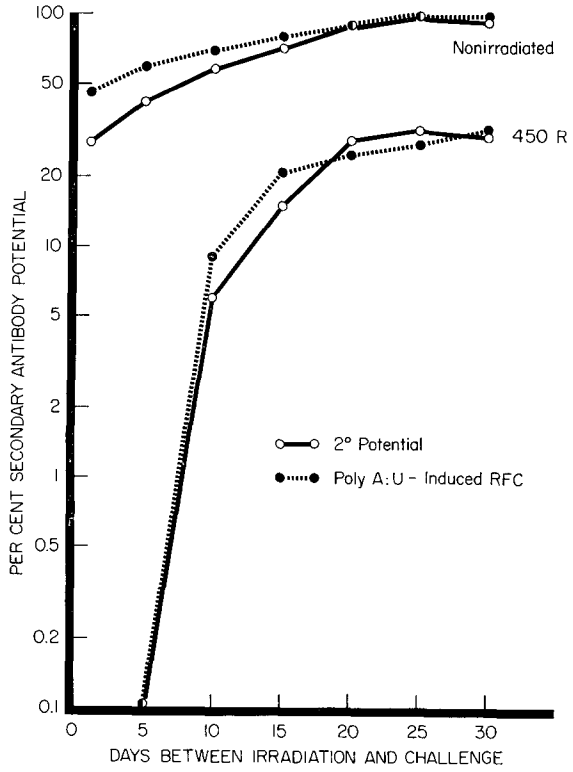

FIG. 7. Recovery of secondary antibody-forming potential and poly A:U-induced RFC after whole body irradiation. Mice were subjected to 450 R whole body irradiation 2 wk after intravenous injection of 100 μg of HGG. The secondary (2°) potential (○—○) was determined 8 days after intravenous injection of 200 μg of HGG. Recovery of poly A:U-induced RFC (●···●) was determined by assay of spleen cells 24 hr after intravenous injection of 300 μg of poly A:U. All assays were run on pooled spleen cells of three mice per point. The per cent potential was based on a maximum secondary antibody response of 585,000 RFC/spleen, primary response of 93,000 RFC/spleen, and poly A:U-induced response of 84,000 RFC/spleen.

Effect of Poly A:U on Secondary Responsiveness.—On the basis of the above experiments, it appeared that, although the poly A:U-induced RFC were a measure of memory cells (Figs. 6 and 7), the polynucleotide complex neither increased nor decreased the number of memory cells present (Fig. 4). To confirm this, mice were primed by injection of 200 μg of HGG intravenously. 35 days later, a group of mice was given three intravenous injections of 300 μg of poly A:U at 3-day intervals (total dose of 900 μg). The mice were challenged

with 200 μg of HGG intravenously 5 days after the last injection of poly A:U. Another group of mice were challenged with the same dose of HGG 48 days after the first injection of HGG without interim treatment with poly A:U. No significant difference was observed between the secondary responses of mice receiving the poly A:U treatment and those not receiving the poly A:U (Table III).

It had been reported previously that administration of poly A:U 18 hr before antigen suppressed the normal primary response (4). In analogous fashion, the data in Fig. 3 revealed that primed cells were refractory to a second exposure to poly A:U for approximately a 2 hr period after initial exposure to

TABLE III

Attempts to Decrease Immunological Memory In Vivo by Multiple Doses of Poly A:U

System	Days after final antigen injection‖					
	4		8		12	
	RFC/10^6 cells	Titer	RFC/10^6 cells	Titer	RFC/10^6 cells	Titer
1° Response*	2300 ± 343	10	5800 ± 864	160	5200 ± 645	160
2° Response‡	7500 ± 744	640	18900 ± 1865	20480	11700 ± 1450	10240
2° Response§ + poly A:U	6600 ± 818	1280	17800 ± 1766	10240	12600 ± 1386	20480

* Mice received a single intravenous injection of 200 μg of HGG.

‡ Mice received 200 μg of HGG intravenously 49 days after the primary injection of 100 μg of HGG intravenously.

§ Mice received 100 μg of HGG intravenously; 35 days later, they received three 300 μg doses of poly A:U intravenously at 3-day intervals; 5 days later, they received 200 μg of HGG intravenously.

‖ Numerical values represent arithmetic mean of 3 mice; ± values indicate the 95% confidence limits.

poly A:U. An investigation was undertaken therefore to determine whether primed spleen cells were responsive to specific antigen during or after that refractory period. To this end, an adoptive system was employed. Primed spleen cells were cultured in the presence of 10 μg/ml of HGG for 1 hr and washed three times in PBS, pH 7.2. 2.5×10^7 cells were injected intravenously along with 10^7 freshly prepared normal spleen cells into lethally irradiated (800 R) isologous recipients. The adoptive response elicited in this manner was first detectable at 4 days and peaked 8 days after transfer (Fig. 8). No response was detectable in recipients of cells that were not incubated with antigen. Irradiated recipients receiving no cells usually died within 8 days. 50% of the animals receiving cultured primed spleen cells without 10^7 freshly prepared normal spleen cells died within 6 days; the remaining died within 10 days. 95% of the

mice receiving normal spleen cells survived at least 8 days. Incubation of normal spleen cells for 1 hr with HGG was not sufficient for induction of a primary response; the sera and spleens of recipients of such cells were uniformly negative.

With the above system established, the ability of spleen cells from primed mice to respond to antigen after culture in poly A:U was determined. Accordingly, spleen cells were cultured for 1 hr in 10 μg/ml of HGG either immediately or 5 hr after culture for ½ hr in the presence or absence of 150 μg/ml of poly A:U. 2.5×10^7 cultured cells were then injected intravenously along with 10^7

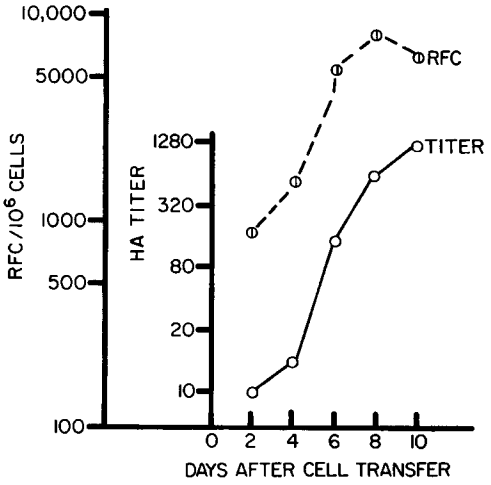

FIG. 8. Kinetics of the adoptive secondary antibody response initiated in vitro to human gamma globulin. Primed spleen cells were cultured for 1 hr in the presence of 10 μg/ml of HGG and then washed three times in PBS 7.2. 2.5×10^7 cultured cells were injected intravenously along with 10^7 freshly prepared, untreated, normal spleen cells into lethally irradiated (800 R) isologous recipients. At intervals after cell transfer, spleens and serum from three mice were collected, pooled, and assayed for anti-HGG RFC and anti-HGG antibody, respectively. Spleens and serum of mice receiving primed cells not exposed to antigen or normal (nonprimed) spleen cells cultured with antigen were consistently negative (<300 RFC/10^6 cells; serum anti-HGG titers <10).

freshly prepared normal spleen cells into lethally irradiated recipients. An antibody response was evident after culture of primed spleen cells for 1 hr with antigen (Table IV). This response was significantly depressed if the cells were cultured for ½ hr in poly A:U but not if the cells were cultured for 5 hr after exposure to poly A:U before the 1 hr culture with HGG (Table IV). No response was transferred by cells not cultured with HGG.

In light of the above experiment it was reasoned that the inability of cells to respond to antigen after ½ hr exposure to poly A:U might be due to release of a cell-associated factor into the culture supernate with subsequent loss upon centrifugation and washing. An attempt was made therefore to restore respon-

siveness to poly A:U-treated cells by culturing the cells in the presence of supernatant fluids from poly A:U-stimulated cells. Spleen cells, from mice injected with 200 μg of HGG 6 wk previously, were incubated in BME for 3 hr in the presence or absence of either 150 μg/ml of poly A:U or 1 μg/ml of HGG. The cells were sedimented and the supernatant fluids collected and held at 4°C. Freshly prepared pools of similarly primed spleen cells were cultured for ½ hr in BME in the presence or absence of 150 μg/ml of poly A:U, centrifuged, washed, and an aliquot resuspended in each of the supernatants described above. After 10 min incubation at 37°C, HGG was added to the cultures to a final concentration of 10 μg/ml and culture continued for another hour. The cells were sedimented and washed three times in PBS, pH 7.2, and

TABLE IV

Effect of Poly A:U on the In Vitro Initiation of a Secondary Response

Culture interval between poly A:U and antigen treatment	Exposed to antigen	½ Hr culture treatment			
		BME		Poly A:U	
		RFC/10^6 cells	Titer	RFC/10^6 cells	Titer
None	−	280	<10	360	<10
	+	7630	640	2990	80
5 hr	−	n.d.	n.d.	320	<10
	+	7910	1280	7180	640

Spleen cells from primed mice were cultured in BME or poly A:U for ½ hr. The cells were washed and exposed to antigen (HGG) for 1 hr either immediately or after a 5 hr culture interval in BME. 2.5×10^7 cultured cells + 1×10^7 freshly prepared, nontreated normal spleen cells were injected intravenously into lethally irradiated (800 R) recipients. RFC and serum titers were determined by assay of pools of spleens or serum from three mice 8 days after cell transfer. Concentrations: poly A:U, 150 μg/ml; HGG, 10 μg/ml.

n.d. = not done.

1.5×10^7 cells were injected intravenously along with 10^7 freshly prepared normal spleen cells into lethally irradiated mice (800 R). The spleens and sera of the recipients were assayed for RFC and antibody, respectively, 8 days after cell transfer.

Pretreatment of the cells for ½ hr with poly A:U before exposure to antigen caused a significant depression of both the RFC/10^6 cells (64%) and serum antibody (2 $\log_2$ units) (Table V). Exposure of these cells to supernatant fluids from either poly A:U- or antigen-treated cultures reversed this inhibitory effect and resulted in a stimulation by antigen of the secondary response as measured by both RFC/10^6 cells (1.7-fold increase over control) and by serum antibody titer (2 $\log_2$ units increase over control) (Table V). Neither excess poly A:U nor the supernatant fluids from nonstimulated cells affected the poly A:U-induced suppression of secondary responsiveness.

To determine the specificity of the stimulatory activity of the supernatant fluids, the ability of such fluids from BSA-primed cells to restore responsiveness to poly A:U–treated HGG-primed cells was investigated. Spleens were removed from mice 6 wk after intraperitoneal injection of 3 mg of BSA. The spleen cells were cultured 4 hr in either BME or BME containing 150 μg/ml of poly A:U. The supernatant fluids were collected and held at 4°C. A separate preparation of spleen cells then was obtained from mice primed to either HGG or BSA. These cells were stripped of their receptors by incubation at 37°C for ½ hr in the presence (or absence) of 150 μg/ml of poly A:U. Control cultures without

TABLE V

Reversal of Poly A:U–Induced Inhibition of Secondary Responsiveness by Supernatant Fluids from Poly A:U or Antigen-Stimulated Cultures

Supernatant* added	HGG added	½ Hr culture treatment‡			
		BME		Poly A:U	
		RFC/10^6 cells§	Titer	RFC/10^6 cells§	Titer
bmeS	−	n.d.	n.d.	420	<10
bmeS	+	5970	160	2380	40
hggS	−	990	<10	370	<10
hggS	+	7320	160	9680	640
p(a:u)S	+	8170	320	10500	640
Poly A:U‖	+	n.d.	n.d.	1790	40

* Primed spleen cells were incubated 3 hr in BME in the presence or absence of either 150 μg/ml of poly A:U [p(a:u)S] or 1 μg/ml of HGG (hggS). The supernatant fluids of these cultures were used as culture medium for the culture of a separate preparation of primed cells.

‡ Cells were cultured ½ hr in the presence or absence of 150 μg/ml of poly A:U, sedimented, and resuspended in the appropriate supernatant fluid. The cells were then cultured for 1 hr in the presence (+) or absence (−) of 10 μg/ml of HGG.

§ 1.5×10^7 cells + 10^7 freshly prepared normal spleen cells were injected into lethally irradiated (800 R) recipients. RFC and serum titers were determined 8 days after cell transfer by assay of the pooled spleens or serum from the five mice in each group.

‖ 150 μg/ml of poly A:U was used rather than a supernatant fluid.

poly A:U were included. The cells were washed and resuspended in one of the supernatant fluids prepared earlier. After 10 min at 37°C, either 10 μg/ml of HGG or 60 μg/ml of BSA was added to the cultures. 1 hr later the various cell suspensions were washed and injected into separate groups of lethally irradiated (800 R) mice. The serum and spleens of the recipients were collected and assayed 7 days after cell transfer.

As may be seen in Table VI, the exposure to HGG of HGG-primed cells cultured in control supernatant fluids (bmeS) resulted in an anti-HGG antibody response. Exposure of such cells to BSA, whether cultured in bmeS or BSA-primed cells cultured in poly A:U–stimulated supernatant fluids [$p(a:u)S_{bsa}$], did not result in a significant anti-HGG response. Similarly, exposure of BSA-

primed cells to BSA resulted in an anti-BSA antibody response. Pretreatment of the cells for ½ hr in poly A:U before exposure to antigen in bmeS reduced both the anti-HGG and anti-BSA responses. Exposure of poly A:U–treated, BSA-primed cells to BSA in the presence of $p(a:u)S_{bsa}$ resulted in full restoration of the anti-BSA response. The $p(a:u)S_{bsa}$ did not restore the responsiveness of poly A:U–treated HGG-primed cells to HGG. However, the exposure of poly A:U–treated HGG-primed cells to BSA in the presence of $p(a:u)S_{bsa}$

TABLE VI

Specificity of Supernatant Factor Responsible for Reversal of Poly A:U–Induced Suppression of Secondary Responsiveness

Cells primed with	Antigen added	Supernatant added	½ Hr culture treatment							
			BME		Poly A:U		BME		Poly A:U	
			Anti-HGG		Anti-HGG		Anti-BSA		Anti-BSA	
			RFC*	Titer	RFC*	Titer	RFC*	Titer	RFC*	Titer
HGG	HGG	bmeS	6550	160	2280	40	397	<10	285	<10
		$p(a:u)S_{bsa}$	8820	320	3370	40	163	<10	309	<10
HGG	BSA	bmeS	570	<10	416	<10	317	<10	168	<10
		$p(a:u)S_{bsa}$	952	<10	2160	20	570	<10	168	<10
BSA	BSA	bmeS	347	<10	212	<10	4760	80	1980	20
		$p(a:u)S_{bsa}$	480	<10	620	<10	6130	160	7340	160

Spleen cells from mice primed to either BSA or HGG were cultured ½ hr in either BME or BME + 150 μg/ml of poly A:U, washed, and resuspended in supernatant fluids from cultures of BSA-primed cells incubated 4 hr in either BME or 150 μg/ml of poly A:U. 10 min after resuspension, either 60 μg/ml of BSA or 10 μg/ml of HGG was added to the cultures and incubation was continued for 1 hr. 10^7 cultured cells and 10^7 normal spleen cells were injected intravenously into lethally irradiated (800 R) recipients. 7 days later, spleens and serum from five mice per group were collected, pooled, and assayed for anti-HGG and anti-BSA RFC and serum antibody.

* RFC = RFC/10^6 cells.

resulted in a significant anti-HGG response, even though the cells were not exposed to HGG again (Table VI). Similar exposure of poly A:U–treated HGG-primed cells to BSA in the absence of $p(a:u)S_{bsa}$ did not result in a significant anti-HGG response. In no instance were HGG-primed cells observed to produce anti-BSA nor were BSA-primed cells observed to produce anti-HGG.

To determine whether the activity of the supernatant fluids described above was due to the presence of specific immunoglobulin released from the cells during culture in the presence of poly A:U, supernatant fluids obtained by culture in the presence of 150 μg/ml poly A:U of BSA-primed [$p(a:u)S_{bsa}$] or

HGG-primed [p(a:u)S_{hgg}] cells for 4 hr were treated with either GAM7SGG or NRS and then assayed for ability to either restore the response of poly A:U–treated HGG–primed cells upon exposure to HGG or, in the case of p(a:u)S_{bsa}, to allow the induction of an anti-HGG response upon exposure to BSA. The protocol used for Table VI was followed with the addition of a 45 min incubation of the supernatant fluids at 37°C with either 1:200 NRS or 1:800 GAM7SGG. The exposure of control HGG-primed cells to HGG in the presence of p(a:u)S^{hgg} resulted in a slight increase in the anti-HGG response (Table VII). Treatment of the supernatant fluids with 1:800 GAM7SGG reduced this response but not significantly below the re-

TABLE VII

Neutralization of Supernatant Fluids with Anti-7S Gamma Globulin Serum

Supernatant added	GAM7SGG	Antigen added	½ Hr culture treatment			
			BME		Poly A:U	
			RFC/10^6 cells	Titer	RFC/10^6 cells	Titer
bmeS	−	HGG	8320	320	3420	80
p(a:u)S_{hgg}	−	HGG	9630	640	10130	640
p(a:u)S_{hgg}	+	HGG	6670	160	3070	40
p(a:u)S_{bsa}	−	BSA	n.d.	n.d.	2880	40
p(a:u)S_{bsa}	+	BSA	n.d.	n.d.	984	<10

Supernatant fluids were prepared by culturing cells obtained from mice primed to either HGG [p(a:u)S_{hgg}] or BSA [p(a:u)S_{bsa}] 4 hr in the presence or absence of 150 μg/ml of poly A:U. Portions of the supernatant fluids were incubated 45 min with either 1:200 NRS (−) or with 1:800 GAM7SGG (+). Spleen cells from mice primed to HGG were treated according to the text. 2×10^7-treated cells were injected into irradiated (800 R) recipients. Spleens and serum were collected from five mice per group 7 days after cell transfer and assayed for anti-HGG RFC and serum antibody.

n.d. = not done.

sponse of cells exposed to antigen in the presence of bmeS (1 $\log_2$ unit serum antibody; 20% reduction in RFC/10^6 cells). The response of poly A:U–treated cells to antigen in the presence of p(a:u)S_{hgg} treated with GAM7SGG was significantly reduced (3 $\log_2$ units serum antibody; 71% reduction in RFC/10^6 cells). Similarly, the ability of p(a:u)S_{bsa} to allow the elicitation by BSA of an anti-HGG response was abolished by treatment of the p(a:u)S_{bsa} with GAM7SGG (Table VII).

DISCUSSION

Cell-associated antibody in suspensions of primed lymphocytes has been detected by a variety of techniques (12–22). In the work reported here, cell-associated antibody was made manifest by incubation of primed spleen cells with poly A:U. The cell-associated antibody was probably preformed, since it

remained after incubation of the cells for 6 hr in the presence of puromycin. The expression of this antibody was followed by active antibody synthesis after a 2–3 hr latent period. Such a short induction period is plausible since blast transformation and increases in protein synthesis in populations of immune cells have been detected within 4 hr after addition of the transforming agent (23, 24). In addition, there is evidence that chemotactic, cytotoxic, or migration-inhibiting factors are released into the culture medium within 4 hr after recognition of specific antigens by mouse cells (25).

The kinetics of the in vitro and in vivo RFC response induced by poly A:U are compatible with those associated with metabolic changes occurring upon addition of transforming agents (e.g., specific antigen) to immune cells (24). In the latter case, cell division did not occur until 48 hr after addition of antigen, a time at which the in vivo poly A:U–induced response was dissipating. This indication that cell division was not involved in the poly A:U–induced increase in RFC was supported by the observation that mitotic poisons did not affect the response. Since the cells do not appear to divide, the observation that doubel exposure of primed animals to poly A:U resulted in two separate and distinct responses, each displaying similar kinetics and intensities suggested that poly A:U caused a transient increase in protein synthesis without subsequent alteration of the cell's potential for protein synthesis. The protein involved in this case appeared to be a 7S immunoglobulin, since rosette formation by poly A:U–stimulated spleen cells could be inhibited by GAM7SGG serum but not by GAMIgM serum.

Zaalberg et al. observed cells which contained antibody as detected by the fluorescent antibody technique but which did not participate in rosette formation (15). These cells may be analogous to the "memory" cells of Avrameas and LeDuc (20), which have, as a consequence of previous antigen stimulation, retained antibody in the peripheral and/or perinuclear endoplasmic reticulum cisternae. It may be this antibody that is rapidly expressed upon addition of poly A:U to the rosette assay system. "Stripping" the cells of this antibody may result in rapid *de novo* antibody synthesis in a manner analogous to the rapid enzyme synthesis found in some bacteria subsequent to sudden derepression of certain enzyme systems (26). In the latter case, sudden derepression is followed by a rapid burst of enzyme synthesis, resulting in the accumulation of the enzyme in excess of levels normally found within the cells. This "overcompensation" is followed by sharp reequilibration of the rate of enzyme synthesis until normal levels of the enzyme per cell are attained. The rapid, puromycin-sensitive rise in RFC may represent such a phenomenon, the active secretion of antibody being due to an overcompensation with synthesis of more antibody than is required to replace the receptor antibody removed by poly A:U treatment.

The observation that the number of cells responsive to poly A:U could be correlated with the secondary antibody-forming potential attained after

various priming doses of antigen, but not with the intensity of the previous primary response suggests that the cells which respond to poly A:U are specific memory cells. A similar, although more general, correlation has been previously observed between the degree of blastogenesis induced by specific antigen and secondary responsiveness (27). Conversely, the data suggest that memory cells are not formed from cells which participated in antibody synthesis in the previous primary response, a hypothesis for which there is abundant evidence (28, 29). In addition, the parallelism of the recovery of the secondary antibody-forming potential with the recovery of cells responsive to poly A:U with respect to both kinetics and degree suggest that the latter cells are involved in immunological memory.

Only preliminary experiments have been completed on the question as to whether these cells are thymus influenced or bone marrow derived. Nevertheless, rabbit anti-BALB mouse thymocyte serum (6) did not prevent the poly A:U-induced RFC indicating the affected cell was not of thymus origin.

If these cells are responsible for immunological memory, what is the function of the antibody associated with them? Depression of the levels of antibody associated with the cells by incubation for ½ hr in poly A:U resulted in a lessening of the ability of the cells to respond to antigen. Consequently, it is hypothesized that treatment of the cells with poly A:U for ½ hr caused the release of receptor antibody into the culture supernatant. Since the cells were washed before exposure to antigen, this antibody was not associated with them at the time of contract with antigen. When the supernatant fluids of these cultures were added back to the cells, the cells' ability to respond to antigen was restored.

Supernatant fluids from cultures of primed cells stimulated with antigen previously have been shown to enhance the secondary response in vitro (30, 31). There are, however, several differences concerning the nature of the supernatant fluids. Hoffmann and Dutton (32) obtained an active supernatant from macrophage preparations, whereas, in our system increasing the numbers of adherent cells did not enhance the release of receptors. Nakano and Braun (30) proposed that the presence of oligonucleotides was the basis of the activity of their supernatant fluids since they lacked antigen specificity. However, the ability of specific antigens to absorb the active factor (32) and the ability of GAM7SGG to neutralize the activity of the supernatant fluids in the system reported here would suggest that the active factor in the supernatant fluid was a 7S immunoglobulin. Thus, it would seem that suspension of the cells in these supernatant fluids in effect adds back the antibody that was lost from the cells and thus restores responsiveness. Neutralization of the antibody in the supernatant fluids with GAM7SGG either prevents reattachment of the antibody to the cells or prevents the interaction of this antibody with antigen.

It thus appears that cell-associated receptor antibody plays an important role in the induction of the secondary response, and its presence on the cells, is

required for the expression of immunological memory. However, does immunological memory simply involve the acquisition and/or concentration of immunoglobulin receptors? The ability of antigen-specific molecules isolated from antigen-sensitized cells to increase the antigen reactivity of normal cells (33) would indicate that this may be the case. However, the failure of anti-BSA receptors removed from cells primed to BSA to instill secondary responsiveness to BSA in cells not primed to BSA (Table VI) indicates that immunological memory involves more than the acquisition of receptor antibody.

The rigid restriction of cells for antibody class and specificity (27, 28, 34, 35) suggests that the cells have made an internal, possibly genetic, commitment to produce a single type of antibody of predetermined specificity. In our experiments it was observed that when the antibody associated with cells primed to HGG was replaced with anti-BSA receptor antibody, these cells did not produce anti-BSA on exposure to BSA, but rather they did produce anti-HGG antibody. Whether one regards the stimulus, in this case BSA-anti-BSA, as specific or nonspecific, the logical explanation for the synthesis of anti-HGG antibodies in response to BSA is that the cells were internally committed to produce anti-HGG upon stimulation.

It therefore appears that the acquisition of immunological memory involves both the acquisition of receptor antibody and a genetic commitment to produce a distinct antibody of predetermined specificity. It is proposed that upon first contact with antigen, precursor cells are committed, either through selection or instruction, to produce a given antibody. The cells may produce a small quantity of this antibody which becomes associated with the cell. The reaction of antigen with this antibody receptor would act as a trigger for the induction of antibody synthesis. The cells respond only to the antigen to which they are committed since the antibody trigger associated with the cells is directed only against that antigen. Conversely, the cells are capable of responding to any antigen which is reactive with the receptor antibody, regardless of the internal commitment of the cell [cf. doctrine of original antigenic sin (36)]. If the antibody associated with the cell is replaced with an antibody receptor of different specificity, only the specificity of the trigger has changed. The internal commitment of the specificity of the antibody product remains unchanged. Thus, the cells committed to produce anti-HGG could do so in response to BSA since the specificity of the trigger (receptor antibody) had been changed to anti-BSA.

SUMMARY

Addition of polyadenylic-polyuridylic acid in complex form (poly A:U) without antigen to a suspension of spleen cells obtained from BALB/Aj mice primed 6 wk previously with human γ-globulin (HGG) resulted in an immediate fourfold increase over background number of anti-HGG rosette-forming cells (RFC). Culture of similar cells in the presence of puromycin for 1–6 hr before poly A:U did not significantly reduce the response. Continued culture of

primed spleen cells in the presence of poly A:U, resulted in a decrease of RFC to background levels within an hour followed by an increase again 6 hr later. This later increase in RFC was inhibited by addition of puromycin to the culture medium.

The nonspecific stimulation by poly A:U of antibody production by primed spleen cells also was induced in vivo. Increases in splenic RFC were detectable 6 hr after intravenous injection of poly A:U alone, without antigen, into primed mice. The response peaked at 18 hr and had dissipated completely within 3 days. A second injection of poly A:U 24 hr or later after the first injection resulted in a second response, similar to the first with respect to kinetics and intensity. Rosette formation by poly A:U–stimulated cells could not be inhibited by mitotic poisons, but was inhibited by treatment of the cells with goat anti-mouse γ-globulin serum, suggesting that the antibody involved was a 7S γ-globulin.

The decrease in RFC induced by culture of primed cells for 1 hr in poly A:U paralleled a decrease in secondary responsiveness of the cells to antigen. This poly A:U–induced inhibition of secondary responsiveness could be reversed by suspending the treated cells in supernatant fluids derived from poly A:U–stimulated cultures. The reversal was specific in that supernatant fluids removed from bovine serum albumin (BSA)–primed cells by poly A:U did not stimulate the response of HGG-primed cells to HGG. However supernatant fluids from BSA-primed cells caused the production of anti-HGG RFC if BSA rather than HGG was used as triggering antigen. The active factor in the supernatant fluids appeared to be a 7S γ-globulin since activity was lost after 45 min incubation of the supernatant fluids in the presence of goat anti-mouse 7S γ-globulin serum.

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