OLIGOMERIC IGA: THE MAJOR COMPONENT OF THE IN VITRO PRIMARY RESPONSE OF MOUSE SPLEEN FRAGMENTS*

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Studies of the in vitro humoral immune response have been undertaken in the belief that the factors governing initiation and regulation may be more easily manipulated in a culture dish than in an intact organism. Previous work, however, demonstrated some notable differences between in vivo and in vitro responses.

The mouse spleen fragment culture system established by Globerson and Auerbach (1), for instance, does not mount a primary antibody response to some soluble hapten-protein conjugates that are highly immunogenic in intact mice (this paper), whereas the converse is true of α-dinitrophenyl (DNP)[1]-poly-L-lysine (2) and of several of the immunogens used in the present study. It has also been suggested that there is an inverse relation between the in vivo and in vitro response to sheep erythrocytes when different strains of mice are compared (3). Another major contrast has been in the class composition of the specific antibody. It has been difficult to detect immunoglobulin G (IgG) antibody after primary immunization in cultures, although some success has been obtained (4, 5). Immunoglobulin M (IgM) has usually been reported as the sole or predominant component.

We have examined the antibody response of mouse spleen fragments stimulated in vitro by a series of conjugates of the haptenic determinant 3-nitro-5-iodo-4-hydroxyphenylacetic acid (NIP) (6). The culture system has been used extensively (1, 2, 7), yet no detailed study has been presented of the class distribution of the secreted antibodies or of the types of antigens effective in eliciting a primary response.

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[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; CG, chicken globulin; DNP, dinitrophenyl; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; KLH, keyhole limpet hemocyanin; NIP, 3-nitro-5-iodo-4-hydroxyphenylacetic acid; NIP-cap, NIP-ϵ-aminocaproic acid; PBS, phosphate-buffered saline; PFC, hemolytic plaque-forming cells; PLL, poly-L-lysine; SIII, Type III pneumococcal polysaccharide; SRBC, sheep red blood cells.

Materials and Methods

Animals.—Female C57BL/6 mice, 2–3 mo old, were used for the experiments in this study.

Antigens.—Poly-L-lysine (PLL) hydrogen bromide of mol wt in the range 30,000–50,000 was purchased from Miles Laboratories, Rehovot, Israel, polysaccharide SIII from Burroughs-Wellcome Laboratories, Beckenham, England, and keyhole limpet hemocyanin (KLH) was prepared from material obtained from Pacific Bio-Marine Supply Co., Venice, Calif. Chicken globulin (CG) was prepared in this laboratory (T. Imanishi) by salt fractionation (50% saturated solution of $(NH_4)_2SO_4$) from serum.

NIP-CG was prepared by adding 4% NIP-azide (6) in dimethyl formamide to 10–20 mg/ml solutions of protein in 3% sodium bicarbonate. The quantities of hapten added were adjusted until a final conjugation ratio was obtained of 15 mol of NIP per mol of CG. NIP-poly-L-lysine with a substitution of 0.6% of the lysine residues was obtained by the same approach. NIP-KLH was prepared by adding 30 μl of 4% NIP-azide in dimethyl formamide to 6 ml of 1% sodium bicarbonate containing 30 mg of KLH. All reactions were allowed to go overnight at 4°C. Excess free hapten was removed by dialysis against phosphate-buffered saline solution (PBS). Antigen solutions were sterilized by Millipore filtration (Millipore Corp., Bedford, Mass.) and stored at +4°C.

The preparation of NIP-ethylenediamine-SIII (NIP-SIII) was based on the method of Mitchell et al. (8) for DNP-lysine-SIII. 50 mg of SIII was dissolved in 3 ml of water, pH 9.0, and the pH was adjusted to 10 with 0.01 M NaOH. Freshly dissolved cyanogen bromide (25 mg in 1.0 ml of H_2O) was added to the polysaccharide solution, and the "activation" reaction (9) allowed to proceed, with stirring, pH maintained at 10.0–10.5, by addition of 0.1 M NaOH for 7 min. After addition of 0.5 ml of 1 M $NaHCO_3$, NIP-ethylenediamine (10) (33.5 mg in 1.5 ml, pH 8) was added and stirring continued at room temperature for 4 h; then overnight at 4°C. The reaction solution was dialyzed against water previously adjusted to pH 8.0 with $NaHCO_3$. Unbound hapten was further removed by precipitation with potassium acetate-saturated ethanol, and the precipitate was redissolved in water. This step was repeated six times, followed by successive dialyses against 0.05 ethanolamine, 8 M urea–0.1 M borate buffer (pH 8.3), 0.1 M citrate buffer (pH 2.6), 0.1 M carbonate buffer (pH 10.0), and finally against water. The final product contained 0.1 mol of NIP per mol of the aldobiuronic units making up the polysaccharide. This was diluted for use in appropriate dilutions in sterile PBS.

NIP-sheep erythrocytes (NIP-SRBC's) were prepared according to Pasanen and Mäkelä (11) in a 0.12 M carbonate-bicarbonate buffer, pH 9.25, containing 1 vol of 20% washed erythrocytes and 1 vol of 0.4% NIP-azide introduced into the buffer from a 4% solution in dimethyl formamide. The mixture was kept at room temperature for 1 h, then washed and suspended as a 1% vol/vol suspension in sterile PBS.

Culture Technique.—The Millipore filter well technique for organ culture of mouse spleen fragments was used as previously described for the induction of primary responses to sheep erythrocytes (1) and to the 2,4-dinitrophenyl (DNP) determinant (2), except that Eagle's minimal essential medium was used instead of Dulbecco's modified Eagle's medium and that the chick embryo extract was omitted. The spleen fragments, of 1.0 × 1.0 × 2.0 mm dimensions, were prepared with a McIlwaine tissue chopper machine (Mickle Laboratory Engineering Co., Guilford, England). The culture medium was changed every 2 days. Thus, the antibody titer of "6 day medium" designates the antibody content of medium serving for culture between days 4 and 6. Antigen was added in a volume of 0.01 ml of PBS, placed directly on each fragment from a syringe with a 25 gauge needle. NIP-PLL and NIP-SIII were used at doses of 20 and 0.2 μg per spleen fragment, respectively, unless otherwise indicated. All antigens were added to the spleen cultures within 1 h of explantation.

Anti-NIP Antibody Assay.—The preparation of NIP-conjugated bacteriophage T2 and the measurement of haptenated phage inactivation have been described previously (12). The haptenated phage inactivation titers reported in this paper are the reciprocals of the antibody

dilutions in the final incubation mixture of antibody and phage necessary to inhibit 50% of the plaque-forming units.

Antimouse Immunoglobulin A (IgA) Antiserum.—Rabbits were immunized with α-chain purified from MOPC 315 ascites fluid (kindly provided by Dr. H. Wigzell) by a modification of the methods described by Goetzl and Metzger (13). The ascites fluid was dialyzed into pH 8.6 0.2 M Tris-HCl buffer and reduced under mild conditions with 0.01 M dithiothreitol for 1 h at room temperature. The pH was then adjusted to 8.0, and iodoacetamide added to a concentration of 0.011 M. After a further 15 min at room temperature the fluid was dialyzed against pH 8.0 borate-NaCl buffer and applied to a DNP-lysine-Sepharose immunoadsorbent column (14). The DNP-binding myeloma protein was eluted from the column with a 2.5 mM DNP-lysine solution and dialyzed successively against PBS and water to remove free hapten. Finally, the protein solution was concentrated by *in vacuo* dialysis against 1 M propionic acid, and passed through a column of Sephadex G-100 equilibrated with the same solvent. The three peaks of eluted polypeptide chains, monitored by their light absorption at a wavelength of 280 nm, were pooled, concentrated, and then dialyzed against several changes of PBS.

Two rabbits were each immunized with 1.1 mg of protein from the second, heavy chain (15) pool. An identical booster was given 1 mo later, and a final booster consisting of material from the first, or "aggregate" pool, was administered after an additional month. The rabbits were bled at brief intervals thereafter and finally exsanguinated. The antiserum was rendered monospecific for IgA by passage through a mouse IgG-Sepharose 4B immunoadsorbent column.

The absorbed antimouse IgA serum used in these experiments was immunoelectrophoretically monospecific for IgA against both normal mouse serum and the MOPC 315 ascites fluid. On sucrose density gradient centrifugation it exerted no suppressive effect on anti-NIP activity in the fast (IgM) and slow (IgG) velocity peaks from immune culture media or mouse serum even at the lowest dilution tested, 1:10. It did, however, eliminate entirely the IgA anti-NIP antibody of intermediate sedimentation velocity at a dilution of 1:100.

Sucrose density gradient centrifugations shown in the Results section of this article demonstrate the monospecificity of the antiserum with respect to the antibody classes that inhibit the plaque-forming ability of haptenated bacteriophage. In addition, data from this laboratory (M. Kaartinen, unpublished observations) indicate that the antiserum is similarly monospecific in eliminating an anti-NIP antibody peak from rat lymph that sediments between IgM and IgG. Mouse IgA has been shown to cross-react with rat IgA (16).

Rabbit Antimouse IgG Antiserum.—Prepared previously in this laboratory, it was shown to be monospecific for IgG by the same criteria applied to the anti-IgA serum. It derives from rabbits. Sheep antirabbit Ig serum was a gift from Dr. E. Ruoslahti.

Sucrose Gradient Centrifugation.—This method was used for physical separation of IgM, IgA, and IgG components of the anti-NIP antibody secreted into culture media. Medium (0.1 ml) was layered on top of a 10–35% continuous sucrose gradient in PBS (vol 5.4 ml). Gradient tubes were centrifuged at 40,000 rpm for 16.5 h in a Beckmann L3-50 ultracentrifuge using an SW50L swinging bucket rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). 10 fractions were collected from each gradient through a hole made in the bottom of the tube with a needle. The fractions were titered for antibody activity on the same day.

Hemolytic Plaque Assay.—NIP sheep erythrocytes for the plaque assay were prepared by the same procedure as the immunogenic erythrocytes except that the final concentration of NIP-azide was 0.6%. They were prepared immediately before each assay. The extent of coupling was routinely tested by measuring the hemagglutination titer of a standard anti-NIP serum with each batch of NIP-SRBC. Suspensions of lymphoid cells were prepared from cultured spleen fragments as described by Nakamura et al. (17). The plaquing technique was a modification of that developed by Cunningham and Szenberg (18). Briefly, 0.3 ml of 10% NIP-SRBC suspension and 0.1 ml of undiluted guinea pig complement were added to 0.6 ml of Eagle's minimal essential medium. A 0.4 ml aliquot of this mixture was then mixed with 0.2

ml of the appropriate splenocyte suspension, and aliquots of this mixture were pipetted with a Pasteur pipette into slide chambers calibrated to have a capacity of 0.1 ml. The chambers were sealed with Vaseline and incubated for 30 min at 37°C. Plaques were then counted under binocular microscope. IgA indirect plaques were developed by substituting 0.6 ml of the appropriate dilution of rabbit antimouse IgA serum for the 0.6 ml of medium in the NIP-SRBC plus complement mixture. A final anti-IgA dilution of 1:400 gave maximal enhancement of the number of plaque-forming cells (PFC). Some inhibition of direct plaques was detected, and the percentage of inhibition at this dilution interpolated from 4-day cultures responding to NIP-PLL, which do not have indirect plaque-forming cells of the IgA class. Each reaction mixture was assayed in triplicate.

Inhibition of Antibody Activity.—NIP-caproic acid and NIP_{14}-bovine serum albumin (NIP_{14} BSA) were used as inhibitors. The procedures and calculations are described by Sarvas and Mäkelä (12) for inhibition of haptenated phage inactivation and by Imanishi and Mäkelä (19) for inhibition of plaque formation.

RESULTS

Immunogenicity of NIP-Conjugates as In Vitro Primary Antigens.—Spleen fragments were stimulated in vitro with NIP coupled to various carriers: the synthetic polypeptide PLL, the thymus-independent (20) polysaccharide antigen SIII, sheep erythrocytes, the polymeric protein KLH, and chicken globulin. All these conjugates except NIP-CG elicited the production of anti-NIP antibody in vitro. The dose response curves for NIP-PLL and NIP-SIII are shown in Fig. 1. Only the day 4 and day 8 titers are given. The kinetics of the in vitro response is nearly identical for all of the immunogenic conjugates

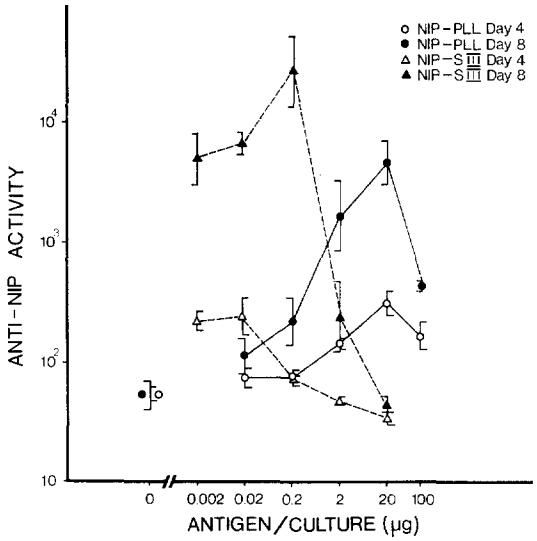

FIG. 1. Antigen dose dependence of the anti-NIP response. ○—○: antigen ε-NIP-PLL, day 4 titer; ●—●: antigen the same, day 8 titer; △---△: antigen NIP-SIII, day 4 titer; ▲---▲: antigen the same, day 8 titer. Vertical bars indicate standard error.

(Fig. 2). Peak titers are found in media collected at days 8 and 10. The data for unstimulated control cultures were pooled and for convenience presented only in Fig. 2 A. In these two experiments each spleen was evaluated separately, and the results obtained from 4 to 10 spleens, mostly by independent experiments, were combined as average titers. The standard errors thus indicate the degree of variation between spleens and at the same time show the degree of reproducibility of independent experiments.

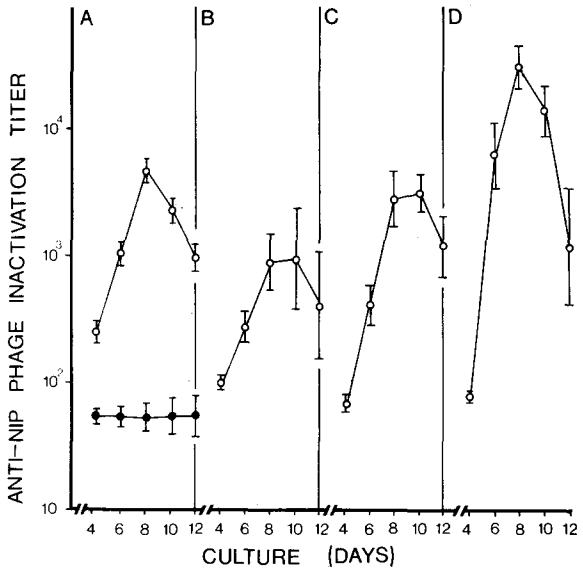

FIG. 2. *A–D.* Kinetics of primary anti-NIP antibody response in vitro. Open circles show average anti-NIP titer in culture media after stimulation with: *A*, ε-NIP-PLL (20 μg); *B*, NIP-KLH (2 μg); *C*, NIP-SRBC (2×10^6); *D*, NIP-SIII (0.2 μg). Closed circles show background anti-NIP titer in unstimulated control cultures. Vertical bars indicate standard error.

The conjugate of SIII elicited the strongest peak (8 day) response as shown in Table I. PLL and SRBC were also effective carriers, whereas a response to NIP-CG could only be demonstrated in one spleen. The poor in vitro immunogenicity of NIP-CG was confirmed by testing a 1,000-fold range of doses. The immunogenicities of NIP-SIII, NIP-PLL, NIP-SRBC, and NIP-CG in vivo were tested for comparison. The "hierarchy" of responses at optimal antigen doses is quite different, most strikingly in the relative positions of the PLL and CG conjugates. The strong in vitro antigen NIP-PLL is quite weak in vivo, whereas the reverse is true of NIP-CG.

Immunoglobulin Classes of Anti-NIP Antibody Produced in the In Vitro Primary Response.—At no time during the 12 days of in vitro primary response studied was a significant amount of 2-mercaptoethanol-resistant antibody pro-

duced (Table I). The antibodies were analyzed further by centrifugation through a sucrose gradient. Fig. 3 shows the gradient profiles of media collected on day 4 and day 8 from the same series of fragment cultures stimulated with NIP-PLL. A rabbit anti-DNP serum was added to the samples before centrifugation as markers for the IgM and IgG positions. The day 4 medium exhibited only a fast-moving component that corresponded in position exactly to that of the marker IgM, whereas the position of antibody from day 8 medium was intermediate between IgM and IgG. Anti-NIP antibodies induced by NIP-KLH, NIP-SRBC, and NIP-SIII had similar sedimentation patterns: the fast-moving component in day 4 media and the predominance of the slower-moving peak in day 8 media.

TABLE I

Antibody Response to NIP Conjugates In Vitro and In Vivo

Antigen	Dose per culture	In vitro titer*		Dose‡ per mouse	In vivo titer			
					7th day		14th day	
		Total	2-ME-resistant		Total	2-ME-resistant	Total	2-ME-resistant
NIP-SIII	0.2 μg	27,300	4.2	10 μg	273,000	5,100	129,000	5,400
NIP-PLL	20 μg	6,000	<3	10 μg	3,900	<90	6,900	477
				100 μg	4,800	<90	51,000	3,600
NIP-SRBC	2×10^5	4,430	6.0	10^8	660,000	48,000	1,470,000	174,000
NIP-KLH	20 μg	1,990	5.7				n.d.	
NIP-CG	20 μg	374§	<3	10 μg	141,000	19,200	9,000,000	2,424,000
None			<3				1,440	<90

Titers of the table were obtained with approximately optimal antigen doses. In vitro titers are log means of 4–10 spleens, in vivo titers are log means of six mice.

* In vitro titers are from day 8 (peak titers).

‡ Subcutaneous injections in complete Freund's adjuvant, except NIP-SRBC, which was injected intraperitoneally in saline solution.

§ NIP-CG is not usually immunogenic for unprimed spleen fragments. These titers are from one mouse, which was an exception, and received 20 μg.

Serum IgA's of most species have sedimentation velocities intermediate between those of IgM and IgG (21). We established the identity of the slow-moving antibody characteristic of primary response culture media by using antiserum specific for mouse IgA in conjunction with sucrose gradient centrifugation. Routinely, 2 μl of antiserum was added to 200 μl of culture medium in a small glass tube. This tube and a control tube containing only culture media were sealed with parafilm, incubated 1 h at an air temperature of 37°C, and then kept at 4°C overnight. 100 μl from each solution was layered on separate sucrose gradients and centrifuged. Gradient profiles show the separation of anti-NIP activity into components of different sedimentation velocities. The anti-IgA serum caused the disappearance of the peak at intermediate velocity (tube 4). This could be due to actual precipitation of IgA by anti-IgA, or to the formation of soluble, inactive complexes that end up at the bottom of the gradient in conditions of antibody excess. Suppression of IgG antibody activity

by this procedure led to the appearance of some active IgG-anti-IgG complexes in the "fast" region of the gradient pattern. Accordingly, a sheep antirabbit Ig serum was added to medium-anti-mouse IgG mixtures after the overnight incubation and incubated an additional hour at 37°C and 4 h at 4°C to assist complex formation.

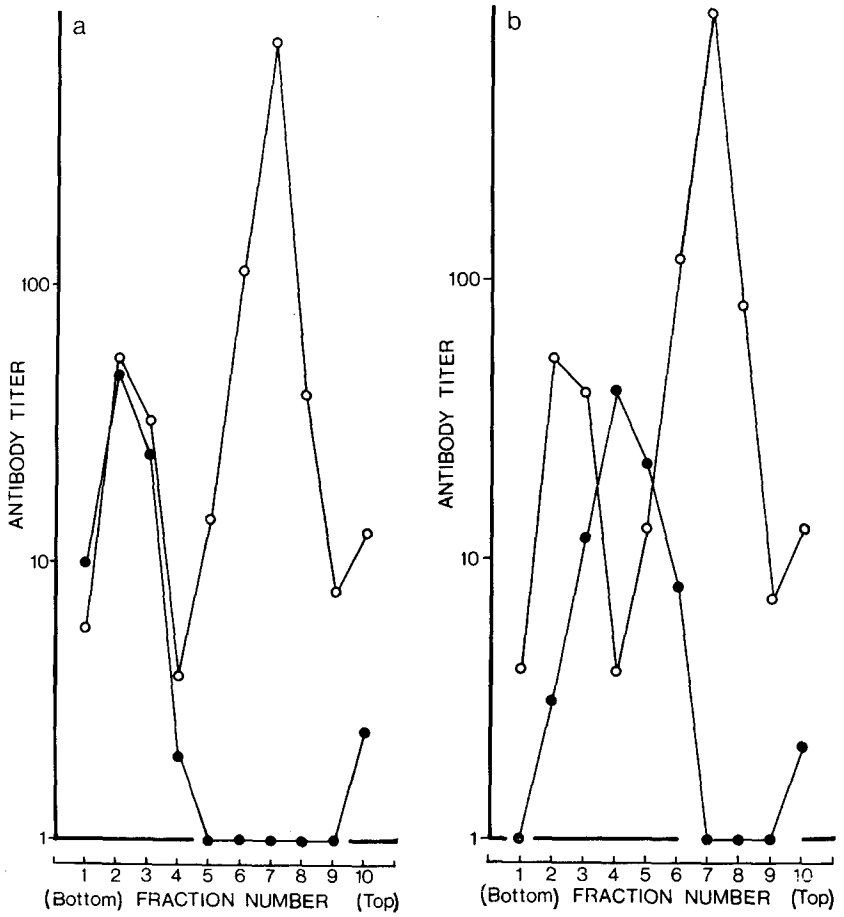

FIG. 3. Sedimentation patterns of anti-NIP antibody. Open circles show anti-DNP activity of a serum mixed into culture medium as a marker before the centrifugation. Closed circles show anti-NIP activity of the culture medium harvested at day 4 (*a*) or day 8 (*b*) of the same culture.

A day 8 anti-NIP-SIII medium (diluted fourfold) was treated with either rabbit anti-IgA or anti-IgG serum. A hyperimmune mouse anti-NIP serum had been added to the medium before treatment as a marker for the IgG position in the gradient profile. The anti-IgA serum completely suppressed the anti-

NIP activity derived from the culture medium (Fig. 4, fraction 4), leaving the IgG marker intact, while anti-IgG serum abolished the marker IgG peak and left the activity from the medium unaffected. Using the above method of

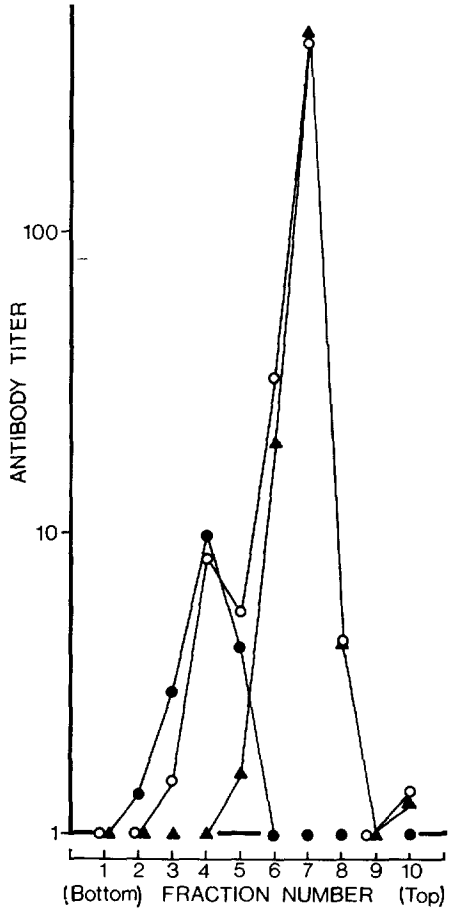

FIG. 4. Effects of preincubation with anti-α or anti-γ serum. ○—○: Control mixture of day 8 culture medium and hyperimmune anti-NIP serum. ●—●: The same preincubated with anti-γ serum. ▲—▲: The same preincubated with anti-α. Fourfold diluted anti-NIP-SIII medium was used.

immunoglobulin class identification we examined in detail the response to NIP-PLL and NIP-SIII. Both IgM and IgA antibodies were detected in vitro, but no IgG. In terms of the time-course of the response, IgM appeared first, and was gradually superceded by IgA. By day 8, most of the antibody activity belonged to the IgA class. The lowest ratio of IgA/IgM sucrose gradient peaks

was 6.5 at that time. Many day 8 media lacked detectable IgM activity altogether.

Immunoglobulin Classes of Antibody Secreted by Hemolytic Plaque-Forming Cells during the Primary In Vitro Response.—As would be expected, anti-NIP hemolytic plaque-forming cells (PFC's) were detected in splenocyte suspensions made from the cultured fragments (Table II). Only direct (IgM) PFC's were present at day 4 of the primary response against both NIP-PLL and NIP-SIII. By day 8, almost all of the PFC's in the anti-NIP-PLL response were at the IgA class, requiring "facilitation" by anti-IgA serum. NIP-SIII induced a longer-lasting IgM PFC response. In some experiments direct plaques still were a major component at the 8th day, but usually IgA PFC's had replaced

TABLE II

IgM and IgA Hemolytic Plaque-Forming Cells from Spleen Explants Immunized In Vitro

Antigen	Time of culture	Anti-NIP PFC/10^6 recovered cells*	
		IgM	IgA
	day		
NIP-PLL (20 μg)	4	2,150	0
" - "	8	150	1,770
" - "	8	50	1,690
NIP-SIII (0.2 μg)	4	4,750	0
" - "	8	27,300	33,200
" - "	8	1,770	8,438
NIP-SIII (0.02 μg)	8	1,698	19,622
" - "	8	2,800	10,000
" - "	8	2,875	20,125

* Each value represents a pool of two spleens from a single experiment.

them by that time. In general, IgM elicited by both the NIP-PLL and the NIP-SIII antigens was seen as a larger component of the PFC response than of the antibody secreted into the media. The direct PFC's at days 4 and 8 may therefore not be secreting antibody as rapidly as the IgA PFC's.

Polymeric Nature of IgA Anti-NIP Antibody Secreted by Spleen Fragments.—The sucrose gradient centrifugation patterns of Figs. 1 and 2 indicated that the IgA molecules secreted by mouse spleen fragments are of a larger size than IgG and are therefore probably polymeric. To establish this in terms of multivalency for binding sites, IgM, IgA, and IgG anti-NIP antibodies from culture media were compared by the method of inhibition of haptenated phage inactivation (12). The superiority of multivalent inhibitors over free hapten is expected to be more impressive the higher the valency of the antibody being tested (12). Table III summarizes the results with NIP-ε-aminocaproic acid and with NIP_{14} BSA as inhibitors. The pattern of avidity for polyvalent and for monovalent

molecules indicates that the valency of the anti-NIP IgA was intermediate between those of IgM and IgG, and resembled more strongly the former. Table IV shows the analogous pattern for inhibition of IgM (direct) and IgA (indirect) PFC's from 8-day cultures.

TABLE III

*Inhibition of Haptenated Phage Inactivation by Culture Media from Spleen Explants Immunized In Vitro**

Antigen	Time of culture	Main immunoglobulin class	HPII‡	
			NIP-cap	$NIP_{14}BSA$
	day			
NIP-PLL	4	IgM	25,333	0.41
NIP-PLL	8	IgA	6,583	0.40
NIP-CG	8 (in vivo)	IgG	534	0.70

* Means of three to five sera or cultures.

‡ Nanomolar concentration of NIP-hapten that reduced the NIP-phage inactivation titer by 50%; each figure is the mean of at least three culture media or sera.

TABLE IV

Inhibition of Hemolytic Plaque-Forming Cells from Spleen Explants Immunized In Vitro

Immunoglobulin class	PFC inhibition*	
	NIP-cap	$NIP_{14}BSA$
IgM	10	0.1
IgA	30	3.0

* Micromolar concentration of NIP-hapten that reduced the number of anti-NIP PFC by 50%.

The sedimentation velocity of IgA in a fresh, unfrozen aliquot at day 8 medium from an NIP-PLL-stimulated culture was identical with that from a repeatedly frozen and thawed aliquot of the same medium as shown in Fig. 5. Thus, the sedimentation of the antibody relative to IgM and IgG is not an artifact of our procedure of storing most media at −20°C before analysis. The same figure allows comparison with the sedimentation velocity of IgA anti-NIP from colostrum of immunized rabbits (22). The mouse spleen IgA was slightly faster than the dimeric (23) rabbit immunoglobulin.

DISCUSSION

The central findings of this report is that oligomeric antibodies of the IgA class are the predominant component of the primary response mounted by

spleen explants against hapten carrier conjugates. This conclusion is based upon the sedimentation velocity of the secreted antibody, which is intermediate between the velocities of IgM and IgG, upon the removal of this predominant component from the sedimentation profile by preincubation with a monospecific anti-IgA antiserum, and upon the high number of IgA plaque-forming cells found in the cultures. IgA is only a minor proportion of the mouse serum immunoglobulins (24, 25) and is not usually detected in the immune response

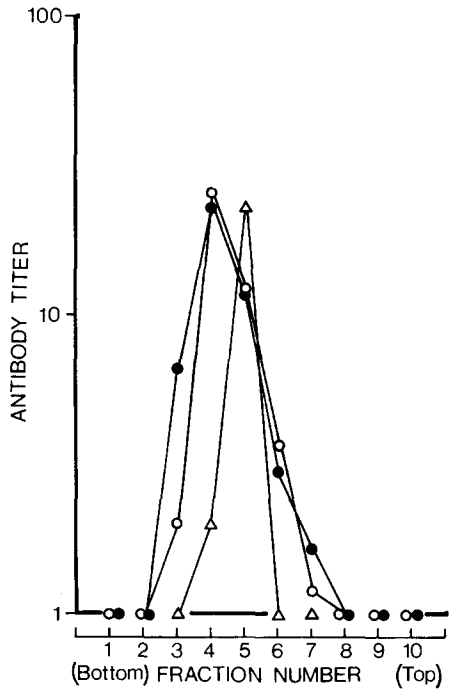

FIG. 5. Size estimation of IgA and the effect of freezing and thawing. ○—○: Fresh day 8 medium from NIP-PLL-stimulated culture. ●—●: The same, three times frozen and thawed. △—△: Rabbit colostrum IgA.

of intact mice. The primary antihapten response by cultured spleen fragments also differed from the response of intact mice with respect to the immunogenicity of the various antigens. In vivo, NIP-CG was very immunogenic and NIP-PLL was a very weak antigen. In culture the reverse was true. Another hapten-protein conjugate commonly immunogenic in vivo, NIP-ovalbumin (OA), also failed to elicit a primary in vitro response (I. Nakamura, unpublished results).

The prevalence of oligomeric IgA in culture was not restricted to those antigens capable of eliciting a primary response. Protein conjugates NIP-OA and NIP-CG could be immunogenic to spleen fragments, but only if these came from mice preimmunized with the carrier (Nakamura et al., unpublished observa-

tions). This response, which was probably T cell dependent, was again predominantly IgA. Therefore the culture system, rather than any particular antigen or helper cell population, seems to be responsible for the preferred IgA production.

The single other observation of IgA antibody production by spleen cells immunized in vitro enumerated a peak response of 45 PFC/10^6 cells, whereas the IgM and IgG peak responses were approximately 2,000 and 700 PFC/10^6, respectively (4). Quantitative comparison of the two studies is difficult, however, because the culture conditions were different. Pierce et al. described the response of cell suspension cultures at 5 days after immunization, a time at which we also found mainly IgM antibody. Nothing was known about the molecular size of the IgA observed in this study. Available data do not therefore argue against oligomeric IgA being an important component in splenic primary responses taking place in culture for more than 4 days.

Our findings are supported by observations that some 23–28% of mouse splenocytes bear surface IgA demonstrable by autoradiography (26, 27). The low level of IgA in mouse serum, approximately one-tenth that of IgG, may reflect its rate of catabolism rather than a lack of cells committed to IgA synthesis. The half-life of mouse serum IgA is about 0.5 days, whereas the half-life of IgG is more than 4 days (28). The same consideration applies to the failure of many investigators, including ourselves, to detect oligomeric serum IgA antibodies in immune responses of intact mice. Kearney and Halliday, however, did detect serum IgA antibody against SIII (29) and hemagglutinating antibody (probably IgA) of a molecular size larger than ca. 200,000 (30). The detection of IgA plaque-forming cells in spleens of immunized mice has only been reported with sheep erythrocytes or *Escherichia coli* lipopolysaccharide as the antigen (31-35). This may be due to a lack of attempts to detect IgA PFC in vivo, or may actually reflect a relative enrichment of antibody-producing cells in the in vitro systems, where the viability of nonproducers is thought to be low. An alternative explanation that has not been excluded hypothesizes feedback regulation of IgA production by IgG antibody. The predominance of IgA in the fragment culture response would thus be a result of the absence of IgG, and the serum IgG of intact mice would suppress IgA PFC and serum levels.

The relative in vitro antigenicities of our NIP-conjugates may be a reflection of their respective abilities to stimulate T cell-independent responses. SIII was shown by Howard et al. (20) to be completely independent of T-B cell interaction in vivo. Conceivably, electrostatic binding between cationic PLL and anionic lymphocyte surfaces might obviate the requirement for T cells in the case of NIP-PLL in vitro, and binding with serum proteins might decrease the likelihood that this conjugate would even arrive at the lymphoid organs of intact mice. Another possibility that must be considered as explanation for the strong antigenicity in culture of NIP-SIII, NIP-SRBC, and NIP-PLL is that these conjugates may be mitogenic, whereas CG and ovalbumin are not. Mito-

gens are known to induce antibody formation in vitro (36), and mitogenicity of a carrier moiety might therefore potentiate a response to the hapten. The fact that SIII has been demonstrated to be a mitogen (37) may support this explanation.

A point that may complicate evaluation of the immunogenicity data is our preliminary findings on the marked strain difference in mice in the capacity to mount a primary response in vitro (I. Nakamura and A. Ray, unpublished). For instance, C57BL/6 and BALB/c but not C3H and CBA strains responded strongly when challenged with the same antigen in the same culture conditions. IgA is also the main component of the BALB/c response. Investigations of both T cell participation and strain differences in the primary response of spleen fragments are now under way.

Antibody responses rich in IgA are generally associated with local immunization of mucosal surfaces (38). There is evidence in mouse (39) and dogs (40), both of whose serum IgA is predominantly polymeric, that the intestinal lymphoid tissue helps to maintain the serum IgA levels. Oral immunization of germfree mice with sheep erythrocytes or ferritin (34, 41) led to the appearance of IgA antibody-producing cells in extraintestinal lymphoid organs according to a time sequence consistent with migration of antigen-stimulated cells from the gut. These data have led to two suggestions: (*a*) that dimeric serum IgA originates from the mucosae, and that the proportion of monomer in the serum may be an index of extramucosal contribution (38), and (*b*) that the extraintestinal IgA-secreting cells found after parental immunization might have met with the antigen in the gut mucosae and hence migrated to the peripheral organs (42). Our data suggest that polymeric serum IgA can be produced by precursors residing in the spleen before the antigen was introduced and together with the data of Pierce et al. (4) they prove that precursors of IgA-secreting cells reside in the normal mouse spleen. This is in agreement with the finding of Craig and Cebra (43) that IgA precursors in Peyer's patches, the enriched source, not only repopulated the gut when transferred into lethally radiated recipients but also localized in the spleen. The autoradiographic data of Bankhurst and Warner (26) and Nossal et al. (27) discussed above are also in agreement with this view.

SUMMARY

The primary antibody response elicited from mouse spleen explants by conjugates of the 3-nitro-5-iodo-4-hydroxyphenylacetic acid (NIP) hapten consisted mostly of the IgA class. Poly-L-lysine, pneumococcal polysaccharide Type SIII, keyhole limpet hemocyanin, and sheep erythrocytes were effective carriers in this system, whereas chicken globulin was not. The anti-NIP response against all of the immunogenic conjugates was detectable in culture media 4 days after explantation and immunization, and reached peak titers by 8–10 days. IgA was identified by sucrose gradient velocity centrifugation in conjunction with the

use of a class-specific antiserum. The media collected at 4 days contained low titers of IgM antibody, whereas the peak response at 8 days consisted almost entirely of IgA.

The primary response IgA secreted by the spleen fragments was characterized as polymeric by its sedimentation rate through a sucrose gradient, and as polyvalent by its drastically greater avidity for $NIP_{14}BSA$ than for free NIP-aminocaproic acid. Its haptenated phage-inactivating activity was abolished by treatment with 0.1 M 2-mercaptoethanol.

These experiments indicate that precursor cells existing in the spleen before primary immunization can give rise to production of polymeric IgA.

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