Douglas Scott Snyder. KINETICS OF SERUM IGA IN RESPONSE TO SALMONELLA FLAGELLAR ANTIGEN IN BALB/C MICE. (Under the direction of A. Mason Smith) Department of Biology, May 1977.

The kinetics of serum IgA concentrations over a period of 28 days were studied in BALB/c mice immunized with flagellin (MON) or polymerized flagellin (POL) from Salmonella milwaukee bacteria. Radial immunodiffusion (RID) was used to quantify IgA and IgM antibody levels in immune sera; passive hemagglutination (PHA) was used to determine serum antibody titers.

Following a 5 day lag period, IgA concentrations had risen to peak levels in MON immunized mice by day 21 and POL immunized mice by day 28. Corresponding agglutinin titers for antisera against both antigens had peaked by day 5. A subsequent decline in serum agglutinin titers was contrasted to elevated serum IgA concentrations. For comparative purposes, serum IgM antibody concentrations were found to reach maximum levels by day 5 which were associated with peak agglutinin titers observed for the same day. A discussion of possible mechanisms concerning the findings is presented.

KINETICS OF SERUM IGA IN RESPONSE TO *SALMONELLA* FLAGELLAR ANTIGEN IN BALB/C MICE

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Joseph G. Boyette

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To My Father

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INTRODUCTION

In recent years immunoglobulin A (IgA) has become the subject of intensive research. It is now well established that two types may be distinguished, a secretory form of IgA (secretory IgA, sIgA) and a serum form (1). SIgA has distinctive antigenic and structural characteristics (2). At the level of the mucous membrane, secretory IgA is known to provide primary protection against infective microorganisms and the entrance of inert foreign macromolecules. Because it is the predominant immunoglobulin class in external secretions with established biologic function, this form of antibody has been extensively studied. In contrast, serum IgA has received far less attention. While representing much less than one-sixth of the total serum immunoglobulins in mice (1), some doubt exists as to its role in systemic immunity. In addition, the relationship between serum and secretory IgA is presently hypothetical. While several workers believe the systems to be independent (3,4), others present theoretical models for common origin (1). Although the direct mode of action involves inactivation or mechanical removal of offending antigens, indirect effector functions remain obscure (1). It has been well established that IgA in either form does not fix complement (2). Possible alternate pathways have been cited (5,6).

It is important to examine the conceptual basis for the design of the following study. The basic unit of flagella is the protein flagellin, or monomer (MON), possessing a molecular weight of 30,000-40,000 (7,8). MON may be prepared by mild acidification of flagella.

This subunit may readily aggregate under proper conditions to form polymer (POL) of striking similarity to the original flagellum. This subunit/complex system allows antigenic challenge by soluble (MON) and particulate (POL) forms bearing similar antigenic characteristics. Extensive work with MON and POL has revealed the former to be thymus-dependent, the latter to be thymus-independent (9). This distinction may be due to substantial differences in size and structural organization.

The BALB/c strain of inbred mice has been shown to possibly have a genetic predisposition that allows the induction of plasma cell tumors by intraperitoneal injection of mineral oil (10). The induced plasmacytomas are known to elaborate large quantities of antibodies, a relatively large number being of the IgA class. One such immunoglobulin has been found to bind Salmonella milwaukee flagellar preparations (11). Through the use of affinity chromatography (by the coupling of MON to sepharose 4B), preliminary data from the author's laboratory suggested immunization with both MON and POL induced antibodies of the IgA, as well as IgM class. The observations that IgA was produced in both the normal and abnormal systems, and that immunoglobulins of both systems bind POL, presented a most intriguing problem.

Since the relationship between the kinetics of antibody titer and IgA response to MON and POL immunization in normal BALB/c mice had not been previously investigated, it became the major objective of this study. Mice were injected intravenously (i.v.) with MON or POL, and bled on appropriate days. Passive hemagglutination (PHA) was used to

determine serum antibody titers; radial immunodiffusion (RID) was used to quantify IgA and IgM levels in mouse sera. The response in normal mice may provide relevant information regarding the myelomatous condition. Also, the utilization of antigens of bacterial origin may ultimately contribute to an understanding of immunity to infection.

MATERIALS AND METHODS

Animals. Ten to twelve week old, female BALB/c mice were obtained from stocks originally maintained at the Laboratory of Cell Biology, National Cancer Institute, Bethesda, Maryland. Mice were fed Wayne laboratory animal food and water ad libitum. All injections were given i.v. utilizing lateral tail veins. Bleedings were accomplished from the orbital sinuses with heparinized Pasteur pipettes, or by cardiac puncture using 25 gauge needles. Blood samples were immediately placed in a refridgerator to reduce hemolysis, allowed to clot thoroughly, and centrifuged to obtain plasmas. All plasmas were frozen at -20° C for storage. New Zealand white rabbits were also given Wayne ration and water ad libitum. Rabbits were used to produce control antisera and anti-alpha antisera (discussed under separate heading).

Antigens. Salmonella milwaukee (H antigens f, g; O antigen 43) were cultured to select for motile organisms (12). Four tubes of motility medium (Motility Test Medium; Difco Laboratories, Detroit, Mich.) were prepared containing small tubes with both ends open, one end extending at least 0.5 cm above the surface of the medium. The insides of two of the smaller tubes were inoculated and the units incubated at 37° C. On day two, culture growth from the outside of the smaller tubes was taken to inoculate the remaining two tubes, as before. On day three, culture growth from the outside of the smaller tubes of the second inoculum was used to inoculate 100 ml of triptocase soy broth (TSB; Baltimore Biological Laboratories, Cockeysville, Md.). Incubation was carried out overnight at 37° C. On day four, the culture was added

to brain-heart infusion agar (BHIA; Difco, Detroit, Mich.) slants in Blake bottles. The cultures were grown at room temperature for 18 hours. Cultures were harvested with 0.4% formalin and centrifuged at 5000 X G for 15 minutes. The resulting pellet was washed with 0.4% formalin and again centrifuged at 5000 X G for 15 minutes. Cells were resuspended in 0.4% formalin and placed in a Waring Blendor for 10 minutes at the lowest speed. The preparation was centrifuged at 5000 X G for 20 minutes to sediment the cells. The supernatant was recovered and subjected to centrifugation at 114,000 X G for 20 minutes. The pellet was resuspended for 30 minutes in a minimal volume of deionized water acidified with 1N HCl to pH 2.0. The preparation was centrifuged at 100,000 X G for one hour. The pellet was discarded and six equivalent volumes of saturated ammonium sulfate were added to the supernatant to be stored overnight at 4° C. This mixture was centrifuged at 25,000 X G and the resulting pellet was resuspended in a minimal volume of deionized water (50 ml). The suspension was dialyzed extensively against distilled water with 0.001% sodium azide (NaN_3) and stored frozen at -80° C or lyophilized as polymer (POL). The quantity of protein obtained was determined by weighing lyophilized material or by utilizing freshly prepared monomer (MON) for Lowry assays (13). MON was prepared by adding 1N HCl to the POL preparation to reach a pH of 2.0 for 20 minutes, followed by neutralization with 1N NaOH (14). The MON preparation could then be used for the Lowry assay or MON injections.

Rabbit anti-flagellin antiserum. Immunizations were initiated by intramuscular (i.m.) injections of 500 µg MON in Freund's complete

adjuvant. One week later 500 µg MON in Freund's incomplete adjuvant were injected i.m. and subcutaneously (s.c.). Booster immunizations were given i.v. in sterile 0.15 M NaCl (saline) according to the following schedule (expressed as weeks after primary injection): 300 µg in 3 weeks, 500 µg in 4.5 weeks, 500 µg in 41 weeks, and 500 µg in 41.5 weeks. One hundred and twenty ml of whole blood was obtained 4 days after final injection by ear bleed. Serum was obtained from the blood sample by the method previously described for mice. Previous ear bleedings had been done at 4 weeks (50 ml), 4.5 weeks (10 ml), and 42 weeks (2 ml, one day before final bleeding).

Rabbit anti-MOPC 467 antiserum. Initial immunization involved the injection of 8.0 mg MOPC 467 (an IgA myeloma protein) in Freund's complete adjuvant i.m. (4 sites). Four days later, 9.4 mg MOPC 467 were injected in Freund's incomplete adjuvant i.m. (2 sites, rear legs) and s.c. (4 sites, shoulders). All subsequent injections were given in saline in the following manner and time (expressed as weeks after primary immunization): 1.0 mg s.c. at 4 sites (3 weeks), 0.5 mg s.c. at 4 sites (4 weeks), 1.0 mg s.c. at 4 sites (4.5 weeks), 1.0 mg s.c. at 4 sites (14 weeks), 1.0 mg s.c. at 4 sites (16 weeks). Forty ml of whole blood were obtained 4 days after the final injection by ear bleed. Serum was obtained from the blood sample by the method previously described for mice. Previous ear bleedings had occurred at 3.5 weeks (2 ml), 5 weeks (50 ml), 5.5 weeks (40 ml), and 15 weeks (40 ml).

Mouse immunizations. Monomer and polymer were injected i.v. at a

concentration of 25 µg / 0.2 ml saline. Two control groups of mice were injected with 0.2 ml saline. Bleedings were made on appropriate days.

Passive hemagglutination. Sheep red blood cells (SRBC) were washed three times in saline via centrifugation with subsequent removal of the supernatant. Cells were resuspended in saline to a 10% volume SRBC / total volume suspension. Two ml aliquots of 10% SRBC suspension were added to conical centrifuge tubes. Various amounts of freshly prepared MON (as 200 µg, 400 µg, and 600 µg) were added to each of two tubes. All tubes were vortexed. One-tenth and two-tenths ml of chromium chloride ($CrCl_3$) at 3.75 mM (from 37.5 mM stock diluted 1:10) prepared at least one week prior to use were added to each set of tubes. Tubes were again vortexed. The mixtures were allowed to stand at room temperature for 20 minutes. Cells were washed by centrifugation with 0.02 M Tris hydroxymethyl aminomethane (Tris; Fisher Scientific Co., Fairlawn, N.J.) -buffered saline (TBS) with 5% fetal calf serum (FCS; Microbiological Associates, Bethesda, Md.), followed by two washes with balanced salt solution[1] (BSS). Cells were then resuspended to 0.5% volume SRBC / total volume and tested against the control serum for coupling effectiveness (15). Microtiter analysis was performed to assess the effectiveness of coupling. Using calibrated micropipettes, 25 µl of BSS with 5% FCS were introduced into each well of the microtiter plate (Cooke Engineering, Arlington, Va.). Microtitrators calibrated to deliver 25 µl of fluid were dipped into rabbit anti-

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[1]Balanced salt solution: 0.1369 M NaCl, 0.0051 M KCl, 0.0007 M Na_2HPO_4, 0.0056 M dextrose, 0.0248 M Tris, 0.0009 M $CaCl_2$, 0.0005 M $MgCl_2 \cdot 6H_2O$; pH adjusted to 7.4 with HCl.

flagellin control serum and placed in the first wells of each row. Mixing of the diluent (BSS with 5% FCS) and test serum was accomplished by rotation, producing a two-fold dilution of antiserum. A drop of the suspension of coupled erythrocytes was added to each well by a calibrated 25 µl microdilutor. Contents of the wells were mixed by gently swirling the plastic tray. Clear tape was placed over the tray to seal the wells to avoid evaporation. The degree of agglutination was read after 2 hours of incubation at room temperature. The highest dilution showing detectable agglutination was taken as the titer. Negative controls (using saline in place of antiserum) were run for each plate (16). To determine response in mice to antigenic stimuli, the prepared mouse sera was used in place of control antisera; other procedural elements were as described.

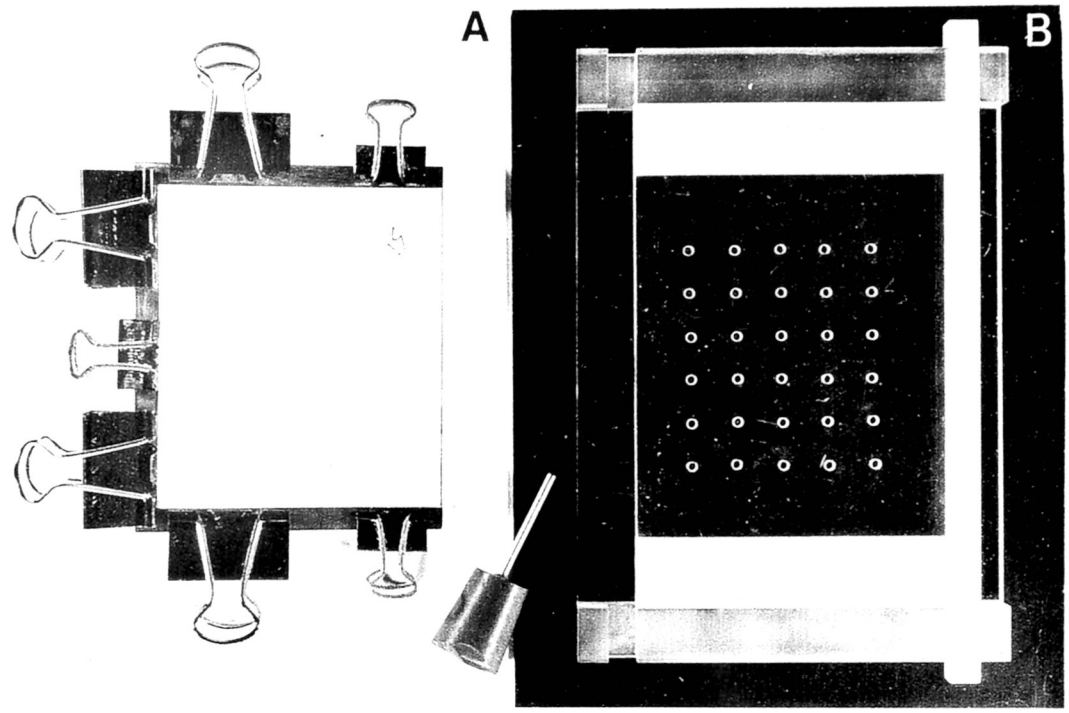
Radial Immunodiffusion (RID). Radial immunodiffusion plates were used to determine concentrations of IgA and IgM in mouse serum. IgM plates were obtained commercially; IgA plates were obtained commercially and prepared in the laboratory. Glass lantern slides (8.1 X 10.0 cm) were purchased to comply with the dimensions of a standard immunoelectrophoresis well/trough cutting frame (17). All top slides were coated with Siliclad (Clay Adams, Parsippany, N.J.) to prevent tearing of the agarose upon removal. All bottom slides were coated with a 1% agarose solution (L'Industrie Biologique Francaise S.A., Gennevilliers, Seine) dissolved in deionized water in a boiling water bath to facilitate adherence of the diffusion agarose to the plate. Base coats were thoroughly dried before use. "U" frames were prepared from brass sheet

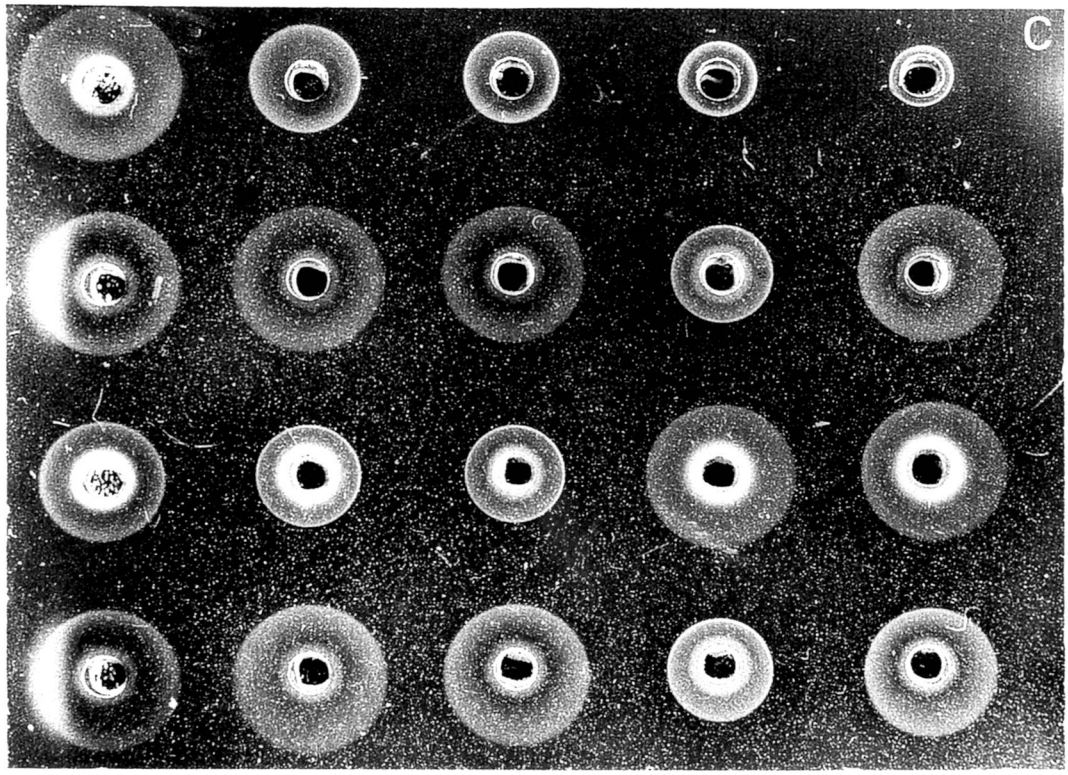
metal of uniform thickness (1 mm) (18). Dimensions corresponded to the lantern slides employed; the arms were 8.1 cm, the base was 10.0 cm, and the brass width of both arms and base equaled 0.5 cm. Edges were sanded to insure uniformity to prevent agarose tear points with top slide removal. Common paper clamps were purchased with attention to the need for firm but not excessive pressure. A plexiglass rectangle as a fitted cover for the immunoelectrophoresis frame was drilled with 30 holes of uniform spacing (1.2 cm from 90° angle origin) with diameters slightly larger than the diameter of the well cutter (inner bore of 2 mm). Uniform positioning of wells allowed optimal ring diffusion (i.e., no interference of adjacent wells, no waste of agarose/antiserum mixture). RID plates purchased from Meloy Laboratories, Springfield, Va. employed antisera raised in goats against immunoglobulins derived from the following myelomas: MOPC 104E for IgM, and a pool of antisera against TEPC 15 and MOPC 315 for IgA. These sera had been cross-absorbed against normal ascites and checked for specificity by Ouchterlony analysis and immunoelectrophoresis by the manufacturer (19). Purified anti-TEPC 15 antisera was obtained through the courtesy of Dr. Michael Potter of the National Cancer Institute. Gamma-globulins of rabbit anti-MOPC 467 were precipitated from the serum adding an equal volume of saturated ammonium sulfate in a gradual drop-wise fashion (20). The precipitate was washed several times by centrifugation and resuspended in the same salt solution. The precipitate was dissolved in water and dialyzed overnight against 0.02 M Tris-HCl, 0.15 M NaCl, pH 8.2. Miniature radial immunodiffusion plates (to conserve

agar and antiserum) were prepared to determine the optimal antiserum dilution (17). Base agarose coats were applied to 1" X 3" slides and allowed to dry. Monospecific antiserum was serially diluted with 0.02 M Tris-HCl buffer, pH 8.2. At high antibody concentrations, ring precipitates were relatively small and intense. With successively lower antibody levels, precipitates became less distinct with increased diameters. Selection of appropriate antiserum dilution is based upon the development of readily visualizable rings over a desired range of antigen concentration. Maximum sensitivity of the assay was reported by Mancini to be 0.00125 mg/ml, with accuracy (18). In this laboratory, accurate measures were limited to 0.05 mg/ml, while retaining reasonably defined precipitates at concentrations up to 5.50 mg/ml. Final dilutions of antiserum were placed in a 37° C water bath to prevent premature cooling of agarose upon mixing. A 2% agarose solution was prepared by melting the agarose in 0.02 M Tris-HCl buffer, pH 8.2 in a boiling water bath. A 1.75 ml volume of thoroughly melted agarose was added to an equal volume of antiserum dilution and mixed rapidly, but gently by inversion. Complete mixing was most important to insure a uniform dilution of antibody throughout the mixture. The 3.50 ml total volume was quickly poured onto the slide, using care to avoid the breaking of surface tension. Slides were placed in a humidifying chamber at 4° C overnight to allow curing. Immediately before use of the plates equidistant wells were cut with the pattern described previously. Plugs were removed by suction using a drawn Pasteur pipette attached to an adjustable vacuum pump. Wells were 2.0 mm in diameter and 1.2 cm

apart, both horizontally and vertically. Serial dilutions of known concentrations of antigen were placed in the wells to establish a working range for each plate (antiserum dilution). Utilizing the small diameter portion of a drawn Pasteur pipette (capillary-type tube with a drawn tip), the wells were filled level to the agarose surface. It was found that a black background with angled incident fluorescent lighting facilitated uniform filling of wells. At well capacity (3.14 µl), no concavity or convexity could be discerned; a shiny smooth surface remained only momentarily as the sample diffused into the agarose medium. Ring development was allowed to proceed for 18 hours at room temperature. The precipitate was viewed using fluorescent light reflected from a black background, giving a dark field effect. The optimal dilution for the desired working range was recorded. Having determined the proper dilution of antiserum to be employed, the RID plate was prepared. The mold was formed by placing the "U" bar between 2 lantern slides, the inner surface of the "top" plate having been previously treated with Siliclad, the inner surface of the "bottom" having received a 1% agarose base coat. The "U" bar and slides were held in place by positioning the paper clamps as depicted in Fig. 1A. Five ml of 2% agarose was added to 5.0 ml of antiserum of optimal concentration. Following rapid, but gentle inversions, the mixture was drawn up into a 10 ml pipette. With the mold held in a slightly slanted position, the tip of the pipette was applied to the slit of the lowered corner. A small portion of the mixture was allowed to drain slowly down the inner edge. The mold was then tilted to permit the fluid to flow across the inner edge of the

Figure 1. Radial immunodiffusion apparatus. A, assembled RID mold. The "U" bar is positioned between two glass lantern slides, the inner surface of the "top" having been previously treated with Siliclad (Si), the inner surface of the "bottom" having received a 1% agarose base coat. The three pieces are held in place by means of clamps; B, modified immunoelectrophoresis frame with plexiglas template inserted. An agarose-covered plate was placed on the base of the frame and the template was fitted above. Antigen wells were cut with the tubular cutter pictured. The agarose plate was then removed from the frame and the agarose plugs aspirated from each well using a drawn Pasteur pipette attached to an adjustable vacuum system; C, appearance of ring diffusion in an IgA antibody-agarose plate after an eighteen-hour incubation period at room temperature and treatment with tannic acid. Five dilutions of a standard serum of known IgA concentration appear along the top row of the plate. Test sera were placed in the remainder of the antigen wells.


A
B


C

bottom. Placing the tip of the pipette on the inner edge of the opposite side, the mixture was allowed to flow gradually as before. Filling the mold in this manner afforded sealing and prevented the inclusion of air bubbles. Depending on the ambient temperature, the temperature of the mixture, and the solidification rate of the gel, care was taken to allow a sufficient seal to develop without premature gelling of the mixture (resulting in a wavy appearance). The agarose-filled mold was placed in a humidifying chamber and cold room (4-10° C) overnight for proper curing. With proper solidification, the clamps were removed carefully, and the top (Siliclad-treated) glass plate was slid in the direction of the bottom of the "U" bar while holding the bar and bottom slide in place. The bar was then removed leaving the gel intact supported by the bottom (agarose base-coated) plate. This plate was placed in the immunoelectrophoresis frame and the plexiglas template was fitted above (Fig. 1B). Antigen wells were punched out and the agarose plugs removed by suction. Wells were filled as previously described. Test sera was run in duplicate. In addition, a set of duplicate reference standards (Meloy Laboratories, Springfield, Va.) was run on each plate. Upon completion of ring development plates were routinely treated with a 1% tannic acid solution for 10 to 15 minutes (21). The tannic acid treatment increased the sensitivity of the assay by increasing ring definition. The precipitate rings could better be observed with the plates held against a black background illuminated obliquely by a fluorescent lamp (Fig. 1C). Ring diameters were determined using a comparator, bearing a magnified scale, held firmly

against the back of the glass slide. A standard curve was prepared for each plate, with standard concentrations plotted on the abscissa and squared values of ring diameters on the ordinate. Squares of the ring diameters of the test sera were then applied to the standard curve to determine sera concentrations.

It should be noted that no bactericidal agent was placed in the prepared gel. There was an indication that sodium azide exerted some effect on the system. In addition, it was found that no agent was required if the gel plates were utilized within several days.

Reduction/Alkylation. A pooling of 9 mouse day 14 anti-POL sera of known titers and IgA concentrations was utilized for this procedure. After thorough mixing, two 0.5 ml aliquots were separated as control and experimental groups. Control and experimental aliquots were dialyzed overnight against 0.15 M Tris-HCl, 0.15 M NaCl, 0.002 M Disodium ethylenediamine tetraacetate (EDTA; Fisher Scientific Co., Fair Lawn, N.J.), pH 8.2 (22). Mild reduction was done by the addition of 0.01 M dithiothreitol (DTT, 1.54 mg/ml; Calbiochem, Los Angeles, Calif.) to the experimental serum followed by incubation for 2 hours at room temperature in the dark. The control sample was treated identically except that saline rather than DTT was added (23). Alkylation was accomplished by adding 0.02 M recrystallized iodoacetamide (IoAc, 4.07 mg/ml; Calbiochem, Los Angeles, Calif.) for 15 minutes at room temperature to both control and experimental groups. Both groups were dialyzed overnight against 0.02 M Tris-HCl, 0.15 M NaCl, pH 8.2. Titers and IgA concentrations were determined for both aliquots.

Statistics. A 2-way analysis of variance with replication, Model III (fixed factor: plates, random factor: mice), was performed to determine if there was any difference due to RID plates prepared from different sources of antisera. Sera from 7 normal mice chosen at random was run in duplicate on each of the 3 types of plates. A 95% confidence level was utilized in the analysis. Means and standard error of the means were calculated for all groups of data. Correlation coefficients were determined to statistically compare IgA level and general titer kinetics for both MON and POL (24).

RESULTS

Dilution of antisera. Optimal dilutions for anti-TEPC 15 and anti-MOPC 467 were found to be 1:32 and 1:64, respectively.

Comparability of RID plates. The results of the 2-way analysis of variance with replication, Model III, are presented in Table I.

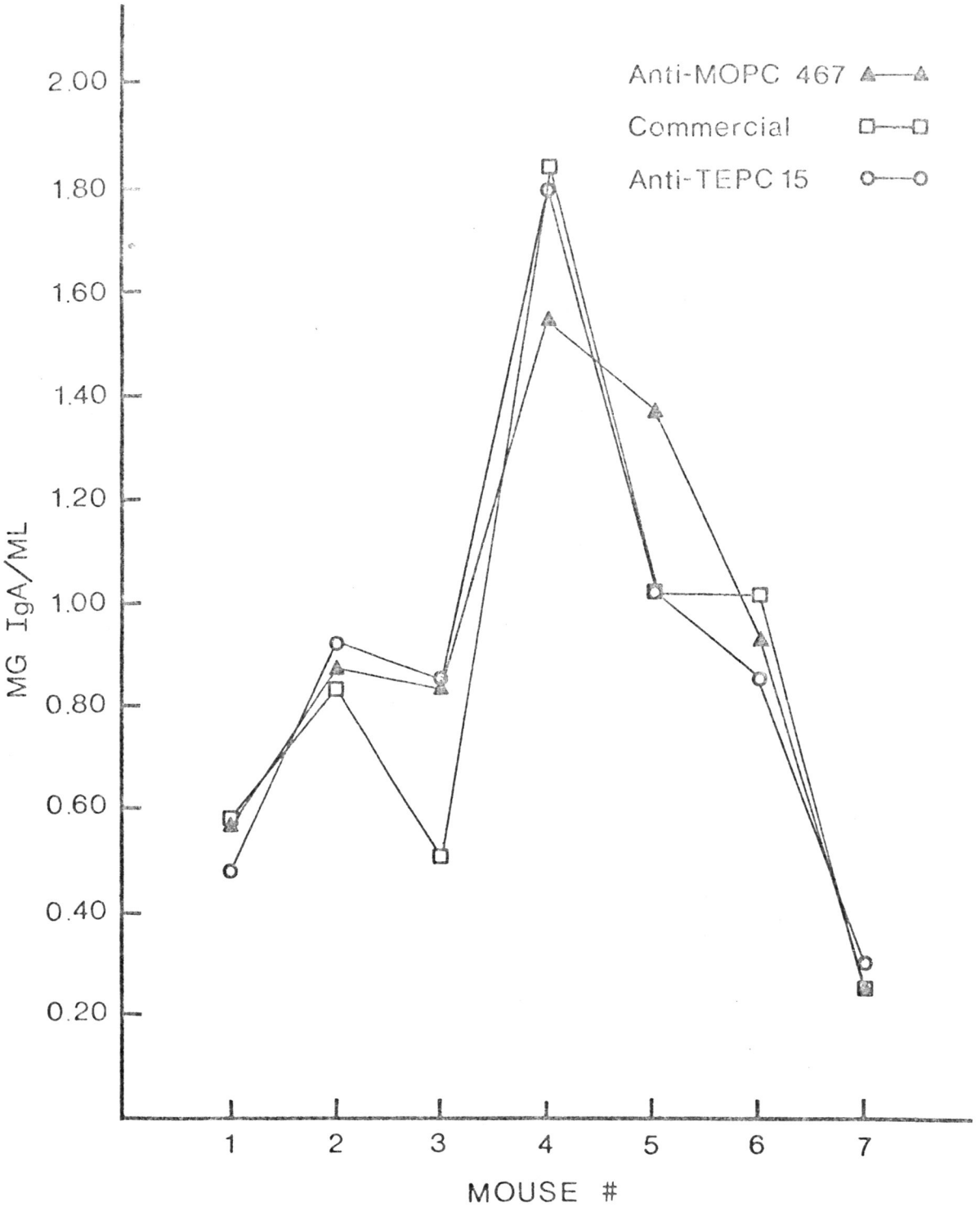
TABLE I

Analysis of variance summary table for comparison of RID plates.

Source of variation	Degrees of freedom	Mean square
Total	41	
Cells	20	
Plates (fixed factor)	2	0.0076
Mice (random factor)	6	1.2995
Interaction (plates and mice)	12	0.0373
Within cells (error)	21	0.0069

It was found that there were no differences in the determinations of IgA concentration among commercial and laboratory-produced plates ($P >> 0.25$). There was a difference in IgA concentration among mice ($P << 0.0005$ concerning the related null hypothesis). There was an interaction of plate type and mice on the IgA concentration determinations ($P < 0.0005$ for the related null hypothesis). Fig. 2 provides graphic representation of results. Sources for significant interaction

Figure 2. Random uninjected mouse IgA concentration determinations for the comparison of three RID plates utilizing different sources of antisera.


Anti-MOPC 467
Commercial
Anti-TEPC 15
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
MG IgA/ML
1
2
3
4
5
6
7
MOUSE #

include mouse numbers 3, 4, and 5.

Data summary table. All data is presented in Table II as arithmetic means of 5-10 animals ± the standard error of the means. Antibody concentrations may be readily compared with related titers for specific day response.

Kinetics of the passive hemagglutination titers of mice immunized with MON and POL. The antibody titers are represented graphically in Fig. 3. No agglutinin activity was detected in unimmunized or saline-injected mice. An agglutinin response was detected shortly after injections of MON and POL forms of flagellar antigen. Between day 2 and day 5 rapid rise in titer of both groups was noted. MON and POL injected mice peaked on day 5, with the MON group showing greater activity. Between day 5 and day 14 titers of MON injected mice fell sharply, reaching the lowest titer on day 21. By day 28, MON associated titers had risen slightly. Beyond day 7, POL injected mice exhibited a gradual decline in serum titers. MON and POL associated titers were comparable regarding rapidity of increase and peak onset. Differences became evident concerning the sharp decline of the MON associated curve, and the gradual decline of the POL associated curve. Beyond day 10, POL injected mice had substantially higher titers than those of MON injected mice.

Kinetics of the concentrations of IgA and IgM in mice immunized with MON and POL. IgA and IgM concentrations were determined for the same sera assayed for agglutinin responses. The serum IgA concentrations are shown graphically in Fig. 4. Unimmunized mice of healthful appear-

TABLE II

Mean[a] IgA concentrations and agglutinin response[b] of BALB/c mice after single immunization with flagellar antigen derived from Salmonella milwaukee.

Assay	Days after antigen injection							
	2	3	5	7	10	14	21	28
Passive Hemagglutination								
Well number Antigen: MON	1.60±0.60	4.00±0.32	7.20±0.20	2.60±0.24	4.50±0.50	2.00±0.55	0.80±0.37	2.00±0.45
Well number Antigen: POL	1.00±0.45	3.00±0.32	5.80±0.37	5.80±0.37	3.00±0.32	5.40±0.24	3.20±1.11	4.40±0.24
Radial Immunodiffusion								
IgA mg/ml Antigen: MON	0.21±0.02	0.21±0.01	0.24±0.02	0.29±0.05	1.80±0.56	1.02±0.25	2.43±0.18	1.38±0.30
IgA mg/ml Antigen: POL	0.14±0.01	0.14±0.01	0.15±0.01	1.06±0.31	1.49±0.48	2.34±0.41	3.13±0.54	3.27±0.64
IgM mg/ml Antigen: MON	0.40±0.03	0.45±0.03	0.56±0.02	0.40±0.01				
IgM mg/ml Antigen: POL	0.29±0.04	0.58±0.08	0.64±0.04	0.25±0.03				

[a]Listed in the table are means for 5-10 mice ± the standard error of the mean.

[b]The results for agglutinin response are expressed as the mean of the last well number containing visible agglutination. The titer can be calculated using the formula 2^n where n = well number.

Figure 3. Passive hemagglutination titers of mice after primary intravenous immunization with monomer and polymer forms of flagellar antigen from S. milwaukee. Each point is the geometric mean titer for a group of 5-10 animals. Data was coded (Well number + 1) for the calculation of the geometric mean.

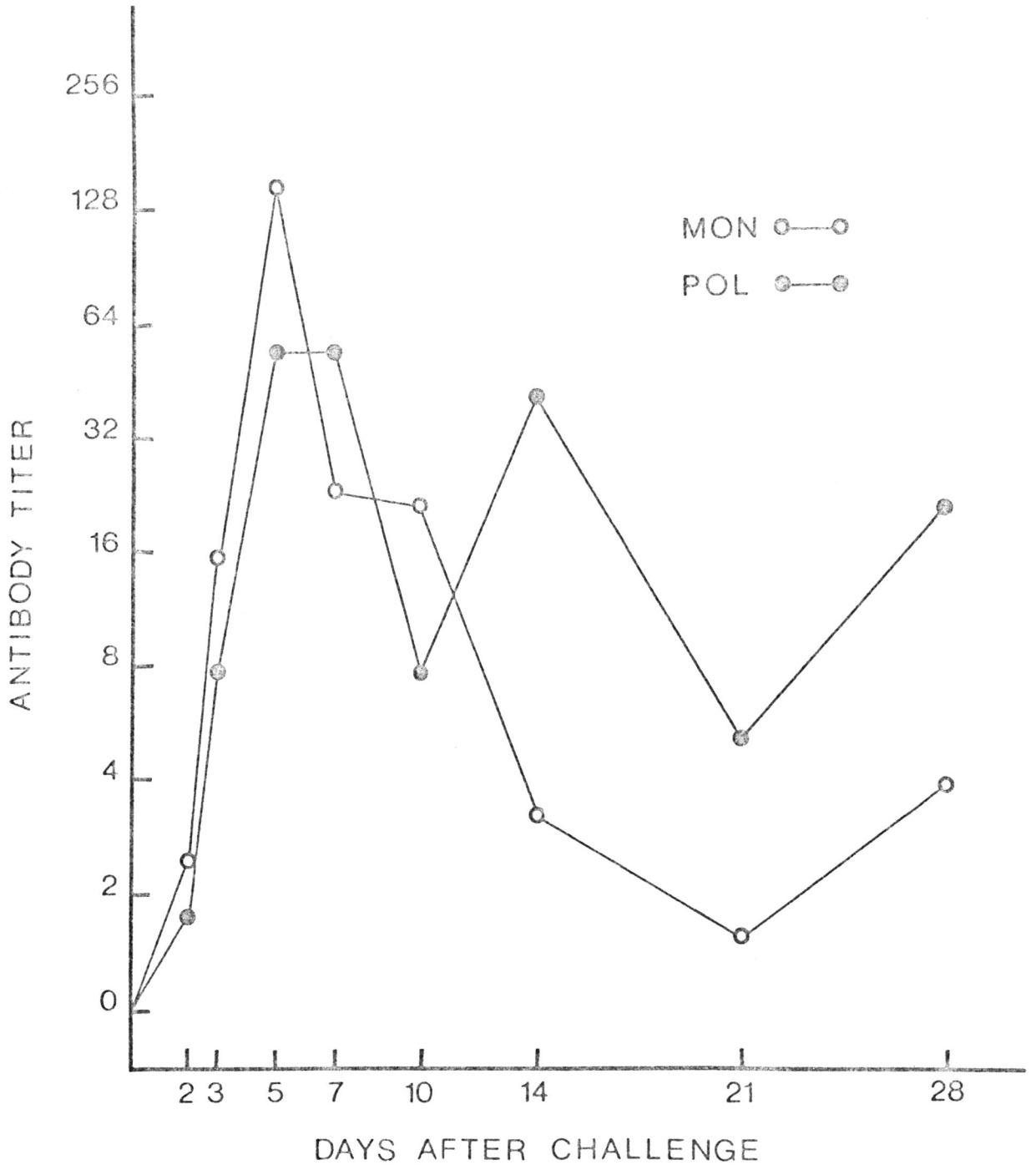
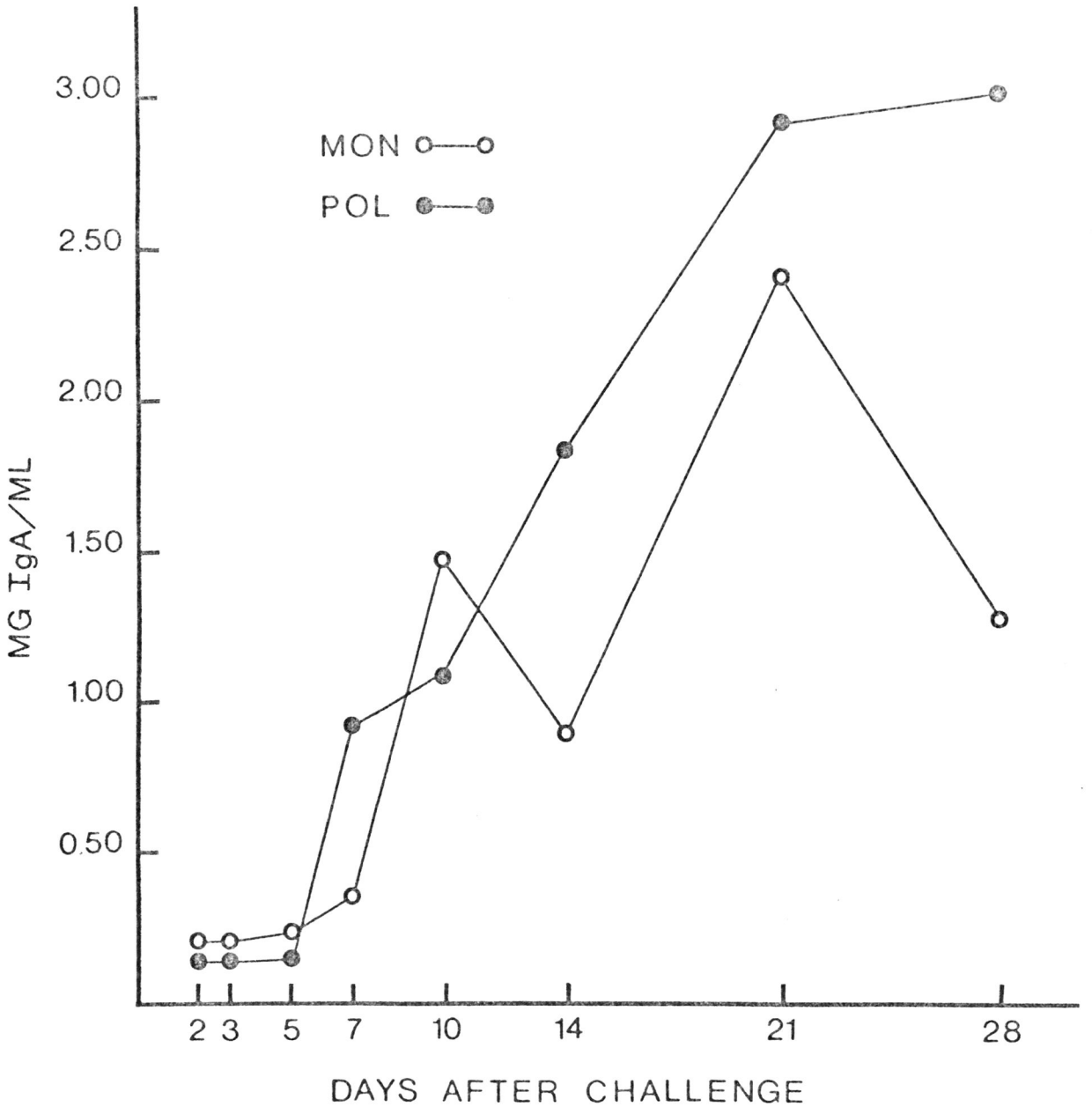
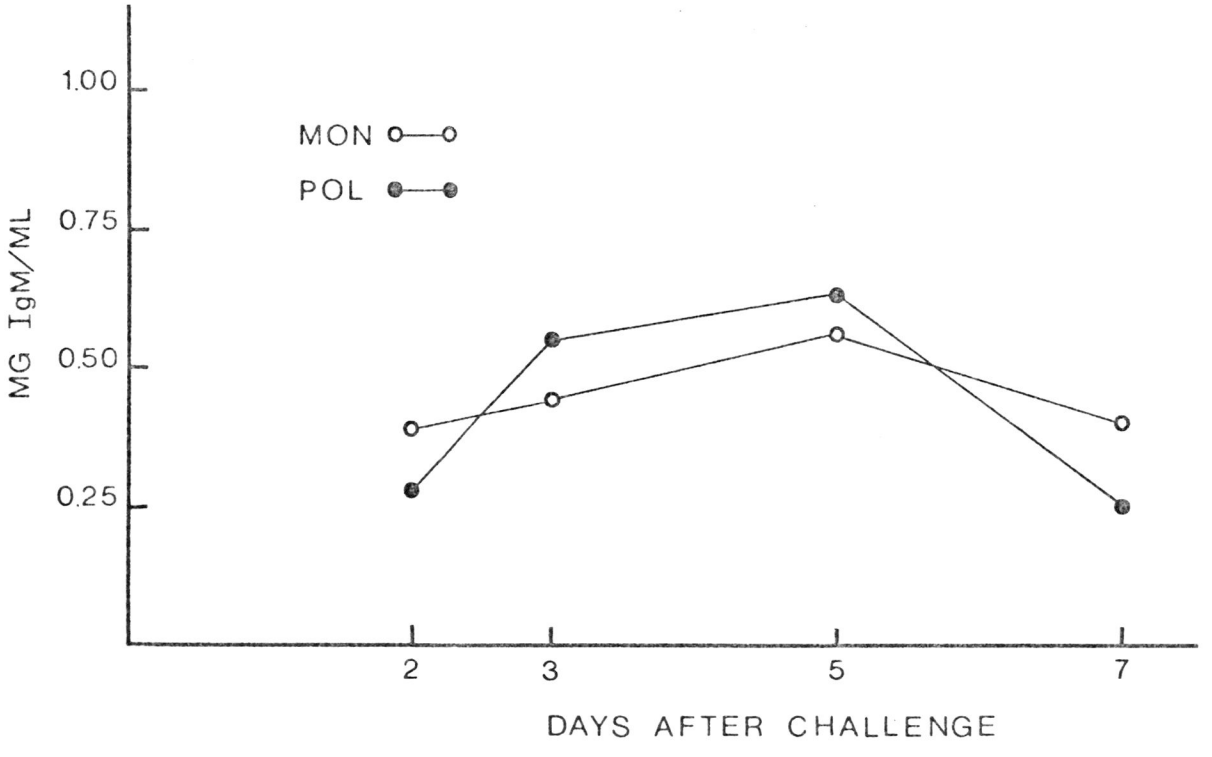

256
128
64
32
16
8
4
2
0
ANTIBODY TITER
MON
POL
2 3 5 7 10 14 21 28
DAYS AFTER CHALLENGE

Figure 4. IgA levels in mg/ml of mice after primary intravenous immunization with monomer and polymer forms of flagellar antigen from S. milwaukee. Each point is the geometric mean concentration for a group of 5-10 mice.


3.00
2.50
2.00
1.50
1.00
0.50
MG IgA/ML
MON
POL
2 3 5 7 10 14 21 28
DAYS AFTER CHALLENGE

ance were found to have a mean serum IgA concentration of 0.18 mg/ml. Mice receiving saline injections had mean serum IgA levels of 0.67 mg/ml and 0.62 mg/ml for days 14 and 28, respectively. Following injection of MON and POL, there was a 5 day lag before any appreciable increase in IgA concentration was noted. Between day 5 and day 7, the POL associated concentration curve exhibited a rapid rise until day 21. Between day 21 and day 28, the IgA levels increased only slightly. A rapid increase to a peak in the MON associated curve had occurred by day 10, with a day 10 IgA level somewhat higher than that of POL. After a slight decline in concentration at day 14, the MON associated curve exhibited a major peak on day 21 which was followed by a decline by day 28. Pertinent differences in the patterns of the two curves include: (a) An earlier increase in IgA concentration against POL than against MON, (b) A steady and rapid rise in IgA levels against POL as opposed to a bimodal response associated with MON, and (c) The substantially higher concentration of IgA to POL on days 14 and 21, with a sustained elevation by day 28. Healthy-appearing, unimmunized mice had a mean serum IgM level of 0.23 mg/ml. The serum concentrations of IgM are shown graphically in Fig. 5. Although relatively low concentrations of IgM were found, definite patterns were detected. POL associated IgM levels increased rapidly between day 2 and day 3, peaked on day 5, and declined by day 7. MON associated IgM concentrations rose to a peak on day 5, and decreased by day 7. A comparison of patterns revealed higher levels of IgM on days 3 and 5 against POL than against MON. On days 2 and 7, MON associated IgM levels were higher than those of POL.

Figure 5. IgM levels in mg/ml of mice after primary immunization with monomer and polymer forms of flagellar antigen from S. milwaukee. Each point is the geometric mean concentration for a group of 5-10 mice.


1.00
0.75
0.50
0.25
MG IgM/ML
MON
POL
2
3
5
7
DAYS AFTER CHALLENGE

Correlation of titer and IgA concentration. Simple linear correlation was employed to statistically examine the relationship between titer and IgA concentration of MON and POL associated curves. For MON injected mice, a correlation coefficient of -0.319 was calculated. POL injected mice had a corresponding value of 0.182. The degree of association between antibody titers and IgA levels in mice immunized with MON and POL is relatively low.

Reduction/Alkylation. The control aliquot had an IgA concentration of 0.82 mg/ml and a corresponding titer of 8. The experimental aliquot had an IgA level of 1.22 mg/ml and a titer of 16.

DISCUSSION

Base concentrations for serum IgA of BALB/c mice have been established by several investigators. Barth *et al.* presented preimmunization IgA levels of 0.7 mg/ml (age range: 2-5 months) (25). Following intraperitoneal injection of 0.5 mg of ferritin and hemocyanin in saline, the BALB/c IgA concentration was found to be 0.4 mg/ml (maximum level obtained 4 to 6 weeks after primary stimulation). For mice chosen at random in my study without attention to healthful appearance, a mean serum IgA concentration was found to be 0.91 mg/ml. Experimental mice carefully selected for healthful appearance had a mean serum IgA concentration of 0.18 mg/ml. This latter value is supported by the findings of Kalpaktsoglon *et al.*, in which the mean serum IgA concentration of BALB/c mice was approximately 0.19 mg/ml for mice 10-12 weeks of age (26). Feeding and housing conditions vary among laboratory animal facilities, thereby introducing variations in antibody levels due to bacterial infestation. The results of my study suggest that 0.18 mg IgA/ml serum is the more reliable value for mice aged 10-12 weeks without obvious signs of illness. It should be noted that aging, itself, apparently has little effect on serum IgA concentrations, since 43 week old normal mice showed a mean serum IgA level of approximately 0.23 mg/ml (26).

A thorough search of the literature failed to reveal comparable studies on serum IgA kinetics in BALB/c mice. However, Langman investigated the response of CBA mice (both sexes, 8-12 weeks of age) injected i.v. with 25 μg POL (15). POL was prepared from *Salmonella typhimurium*,

strain SL870. The author observed a rapid increase in antibody titer which reached a peak on day 7. A slight decline was noted on day 8 with a short rise to a second peak on day 10. Employing a different Salmonella species as an antigen source and a different strain of mice, the mice in my study exhibited slightly lower titers on corresponding days, with peak responses from day 5 through 7 and a decline by day 14. This variance may be ascribed to differences in antigenicities of the flagellar preparations and/or strain response. Barth et al. found hundredfold differences in strain responses with similar antigenic challenge (25). It is also important to note that POL-coupled SRBC were used in Langman's study, whereas MON-coupled SRBC were used as indicator cells in my investigation.

Similar studies have been made concerning the induction of an immune response by flagella, MON, and POL. Possibly, the most important aspect involves breadth of application of results. Various species and strains of test animals have been utilized; sources and preparatory methods of flagellar antigens differ; routes and modes of immunization have varied. With such individualized procedures, the onset of response, the rate of rise in titer, the development of peak titers were dissimilar. There was, however, some basis for comparison. Winebright and Fitch observed an agglutinin response at 4 weeks in rats (27,28). Their findings are represented in my study. Such a prolonged antibody response to the single injection of a protein antigen is considered to be quite unusual. Animals might have been previously exposed to a related antigen (e.g., by a gut-associated organism), such that observed titers

represent a secondary response. This possibility is held untenable by both Nossal, et al. (29) and Winebright and Fitch (27). The absence of antibody activity (whether natural or cross-reacting) in unimmunized mice observed in my study lends some support though limitations in sensitivity of the PHA assay are recognized. Nossal et al. found no anti-H antibody titer without prior immunization of rats (29). Fitch and Winebright reported differences in response patterns to flagella and MON stimulation (28). The rise to peak titers following injection of flagella exceeded that of MON. In contrast, MON was found in my investigation to induce a more rapid rise in antibody titer than POL. The mode of antigen processing for particulate (flagella, POL) and soluble (MON) antigen may be quite different (28), particularly regarding species differences of both antigens and test animals. POL has been found by several researchers to be thymus-independent (9,30), while MON has been reported to be thymus-dependent (9). Davies et al. presented marginal evidence for thymus-independence of flagella (31). Nossal et al. have also found POL-associated peak serum titers were higher than those of MON (29). A proportion of this "excess" antibody contained in anti-POL sera is directed against antigenic determinants created by polymerization itself (7). If titer is somewhat dependent on quaternary structure, two suppositions may be set forth:

1. Anti-POL titers may be under-represented, as MON-coupled SRBC lack in large part (29) the quaternary structure of POL.
2. The elevation of anti-POL titers is not solely dependent upon recognition of antigenic determinants derived from the association of MON subunits.

Therefore, related studies provide a valuable, conceptual framework concerning the immune response to flagellar antigens.

The phasic appearance of POL titers and MON IgA concentrations may reflect a homeostatic regulatory mechanism for antibody synthesis and catabolism (12). Two such mechanisms may operate independently: (a) Immunogenicity of antigenic molecules may decrease as a consequence of combination with antibody. (b) The concentration of antibody may control the rate of antibody synthesis by negative feedback at the cellular and/or synthetic level (32). The serum level of each immunoglobulin class is dependent upon rates of synthesis and catabolism (33). IgA has been reported to have an anabolic rate of 0.64 mg/day/25 g mouse in normal mice. A serum IgA half-life was reported to be on the order of 12 (34) to 24 hours (33). Such a short half-life implicates the interplay of control mechanisms regarding the high levels of IgA detected on day 21 for MON and POL.

Titer and IgA concentration correlation coefficients for MON and POL antigens are -0.319 and 0.182, respectively. These values support visual analysis of the graphed data. Day 5 peak titers for both MON and POL did not include an IgA class directed response (Fig. 4). The delayed but rapid increase in IgA concentration is temporally related to a decline in PHA titers (between day 5 and day 21). It is curious to note that sera containing peak IgA concentrations exhibited minimal titers (day 21). POL induced a higher and more sustained response than MON regarding titer and IgA level. Although the implications of these observations are not firmly established, various plausible explanations

are recognized. There is a possibility that challenge by flagellar antigen induces a non-specific response of the IgA class. Through the use of affinity chromatography in my laboratory, this contention was discounted. Differences in concentration and affinity of immunoglobulin classes could mask IgA class-specific titer. The individualized nature of test results indicates the need for further studies, including IgM and IgG responses with quantification of specific antibody. Grey et al. described an apparent univalency associated with monomer IgA (35). Because a substantial proportion of BALB/c serum IgA occurs in this form (approximately 40%), the PHA titer based upon bivalent (or multivalent) binding could be less than the actual active antibody concentration. Binding of MON at one combining site could place a steric restriction on the binding of MON at the other site. Binding might produce a conformational change of the other combining site, thereby preventing proper association. It has also been proposed that the positions of combining sites (regarding the 3 dimensional structure of the antibody molecule) may vary among molecules. Langman has suggested Salmonella flagellar antigens bear two types of antigenic determinants, designated Hv (variable, i.e. strain specific) and Hc (common, i.e. common to different strains). Antibodies to Hv determinants would have two combining sites on one POL strand, whereas antibodies to Hc determinants would cross-link polymers by binding to one POL strand at a time. The PHA assay was found to preferentially detect antibodies to the Hc determinants (8). As such, some proportion of the antibodies directed against Hv determinants could fail to cross-

link protein strands and reduce apparent agglutinating activity levels. Monomer IgA molecules in the normal system are known to bear 2 combining sites (35).

Because polymer forms of serum IgA diffuse more slowly through agar than monomer (17), it was believed IgA concentration may be greater than that determined (23). Utilizing mild reduction and alkylation procedures to dissociate polymers into monomer form, a 48.8% "increase" in IgA level was determined. Such a difference suggests the presence of a large proportion of polymer forms of IgA in BALB/c mouse sera, as well as the major influence of molecular weight heterogeneity on the radial immunodiffusion assay. The standards used in this procedure were prepared from a mixture of untreated tumor ascites fluids of unknown IgA configuration (19). This "increase" may be misleading in two ways: (a) The objective of the RID assay involved the determination of IgA in mouse sera, not the determination of monomer IgA. (b) Reduction and alkylation procedures may have caused a separation of heavy chains which would give a totally erroneous value. If proportions of monomer and polymer forms of IgA of standards and test sera were equal, diffusion differences would not be problematic. Because the constitution of the standards are unknown, a relative assessment of data may be indicated.

Currently, there is much interest in the possibility of an IgM/IgA shift during an immune response. Although Martin and Leslie found evidence to indicate that IgA-forming cells were derived directly from IgM-forming precursors in chickens, similar studies in mice have been impeded due to the inability of localizing a bursal equivalent (35).

Preliminary data was obtained for an IgM response as a comparison to early kinetic patterns. The peak IgM concentration occurred on day 5, as did peak titers for both MON and POL injected groups. Such a finding implicates IgM contributions to an early immune response. Barth et al. found BALB/c mice IgM preimmunization levels to be 0.8 mg/ml (25), whereas a mean concentration of 0.23 mg/ml was found in my laboratory.

Several subjects demand final consideration. Although the antigen preparatory procedure involved several steps to insure purity, there is considerable doubt if a flagellar antigen alone was injected. Nossal et al. applied special efforts to avoid contamination (29). Highly purified flagellin nevertheless retain "O" antibody activity. It was believed trace quantities of bacterial polysaccharide were not eliminated from the preparation. Vladoianu et al. discovered H and O peptide determinants in flagellar extractions (37). As such, purification based on acid-insolubility alone was not effective. It is well established that MON has a strong tendency to reassociate into POL spontaneously (29). To avoid this change of form, injection immediately followed acidification and neutralization of the POL. Of course, actual configuration upon injection is unknown, and therefore antigenic characteristics of the molecules presented to the immune system are unknown.

Marecki et al. reported that intravenous injection, the route of immunization employed in this study, effected rapid removal of the foreign objects at an early phase of "infection" by the reticulo-endothelial system (38). Alternately, the establishment of salmonellosis by a natural route (i.e., the gut) would delay involvement of humoral

factors. Possibly the lag period of the serum IgA response reflects a presentation of antigen to humoral immune mechanisms withour prior sensitization of a natural challenge. Although it has long been asserted that murine salmonellosis is cellular in nature with humoral elements playing a relatively minor role (39), Marecki et al. present a contradictory view. Specific serum immunoglobulins are believed to function by opsonization of the bacteria. An inflammatory response coupled with the diffusion of phagocytes and protective antibody would be provoked to check extracellular proliferation of the pathogen. The role of serum IgA in systemic immunity has not been well defined. It has been found to be bactericidal in the absence of the complete system, operating through 2 distinct, but unknown factors. Burden has proposed that this class of antibody is important in extravascular spaces from which IgM is excluded (5). Lytic activity reported by Adinolfi et al. (6) is questioned by other researchers due to their inability to reproduce this phenomenon (5). In accord with the designated role humoral elements play in acquired immunity to murine salmonellosis, Wernet et al. report IgA exhibits "powerful opsonic action" which would facilitate ingestion of salmonellae by macrophage (40).

The relationship between serum titers and antibody concentrations has proven to be extremely intriguing. The rapid onset of peak titers to both MON and POL is in sharp contrast to the related IgA "lag." The corresponding peak but low levels of IgM begs the determination of associated IgG levels. Important future studies could utilize affinity chromatography to effectively quantify specific antibody classes and

compare these values to the total circulating concentration of each immunoglobulin class. The kinetics (following primary and secondary stimulation) could aid the delineation of the control mechanisms of response to antigenic challenge by Salmonella flagella. The respective roles of humoral immunoglobulin classes, particularly IgA, may then be clarified.

SUMMARY

Following the immunization of BALB/c mice with MON and POL, there was a five day lag before any appreciable increase in IgA concentration was noted. The monomer associated curve exhibited a bimodal increase, with a peak on day 21. The polymer associated curve exhibited a steady and rapid rise in IgA concentration until day 21, with a slight continued increase by day 28. Mean peak values for MON and POL injected mice were 2.43 mg/ml and 3.13 mg/ml.

MON and POL associated titers peaked on day 5 and days 5 and 7, respectively. These findings corresponded to the lag observed in an IgA concentration increase. Beyond day 5, the MON associated curve exhibited a sharp decline, whereas the POL associated curve exhibited a gradual decline. The decline in antibody titers of both curves is contrasted to elevation in serum IgA concentrations.

IgM concentration curves for both MON and POL peaked on day 5. These peaks corresponded to peak titer determinations, but levels were relatively low.

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APPENDIX

ABBREVIATIONS

Balanced salt solution	BSS
Brain-heart infusion agar	BHIA
Disodium ethylenediamine tetraacetate	EDTA
Dithiothreitol	DTT
Fetal calf serum	FCS
Flagellin or monomer	MON
Immunoglobulin A	IgA
Immunoglobulin M	IgM
Intramuscular injection	i.m.
Intravenous injection	i.v.
Iodoacetamide	IoAc
Passive hemagglutination assay	PHA
Polymerized flagellin	POL
Radial immunodiffusion assay	RID
Secretory IgA	sIgA
Sheep red blood cells	SRBC
Subcutaneous injection	s.c.
Triptocase soy broth	TSB
Tris-buffered saline	TBS
Tris hydroxymethyl aminomethane	Tris