REGULATION OF THE SECONDARY ANTIBODY RESPONSE IN VITRO

II. Chemical Properties of an Antibody Inhibitory Material (AIM) Produced in Antigen-Stimulated Rabbit Lymph Node Organ Culture*

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We have previously described an inhibitor of antibody synthesis found in serum-free medium of rabbit lymph node organ cultures undergoing a secondary response (1). This present paper concerns some chemical properties of this antibody inhibitory material (AIM),[1] notably its sensitivity to ribonuclease. The implications of this latter observation on the isolation of AIM are explored. Other experiments described here indicate that the inhibition of antibody synthesis by AIM is reversible and most pronounced during the productive phase in vitro. Crucial to these studies has been the preparation of media and the recognition of spurious inhibitory effects, particularly those relating to osmolarity and those resulting from the use of certain ultrafiltration membranes. These problems are discussed here. Succeeding papers in this series will deal with the cellular source of AIM[2] and will present evidence for its involvement during antigenic competition in vitro.

Materials and Methods

Culture system.—Our work involved the secondary antibody response induced and followed in vitro. This culture system was originated by Michaelides and Coons (2) and has been extensively modified by us over the past 12 yr (1). Adult male white New Zealand rabbits were primed several months before sacrifice with various soluble protein antigens (bovine gamma globulin [BGG], bovine serum albumin [BSA], or ovalbumin in Freund's complete adjuvant, or alum-precipitated tetanus toxoid or diphtheria toxoid). Each such primed rabbit

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[1] *Abbreviations used in this paper:* AIM, antibody inhibitory material; anti-DT, antidiphtheria toxoid; anti-TT, antitetanus toxoid; BGG, bovine gamma globulin; PHA, phytohemagglutinin.

[2] Ambrose, C. T. 1973. Regulation of the secondary antibody response in vitro. III. Inhibitor-containing cells (ICC) of antigen-stimulated rabbit lymph node organ cultures. Manuscript submitted for publication.

generally provided 1–2 g of lymph node tissue from the popliteal, cervical, and auricular areas. These nodes were cut into cubic millimeter fragments, which were incubated in medium with the priming antigen(s) for 2 h in order to elicit a secondary response. Excess antigen was rinsed away. The fragments were then distributed among Leighton tubes and held in place by pads of glass wool, which when moistened with medium cling to the inner side of the tubes.

In a standard size Leighton tube (16×125 mm^2) with a culture area ("window") of 11×37 mm^2 we placed 14–30 fragments—the number depending on the experimental purpose. These cultures can serve (*a*) as an assay system for testing the presence of an inhibitory effect in a given sample, (*b*) as a source of used ("spent") medium possibly containing the inhibitory material under study, or (*c*) as a source of lymph node fragments from which AIM can be extracted. For assay purposes a convenient range of titers is usually produced by 20 fragments/tube in 8-h periods during the 2nd wk of the response. When cultures merely provided used medium pools as a source of AIM, then 30 fragments/tube were generally used.

All cultures, regardless of their ultimate purpose, received on day 0 and on day 3 the standard medium defined below. Thereafter, the frequency of medium replacement varied. (*a*) Cultures used for assay purposes were usually changed next on day 5, day 6, and then daily, twice a day, or three times a day, as will be indicated in each experiment. The optimal period for assay is from day 7 through day 14, when the secondary response in vitro is at or near a plateau. (*b*) Cultures providing pooled medium generally were changed only every 3 days. The spent medium removed from the cultures of an experiment at any one time was pooled and frozen for storage. Later these 3-day pools were thawed and combined into "early," "mid," and "late" pools, which are defined in each experiment. (*c*) Extracts were also prepared from the lymph node fragments used for assay experiments or for producing pools. These cultures had a prior history of medium changes consistent with their original purpose.

Media Preparation.—Our previous paper (1) described in detail the composition and preparation of the serum-free medium used in earlier studies and reported that the optimal osmolarity of this medium for rabbit lymph node organ cultures is around 280 mosmol/kg. The medium employed in these recent experiments differed in only two respects: (*a*) 10 mM glucose was included instead of the 5 mM formerly added and (*b*) 20 mM HEPES was incorporated to provide a more uniform buffering capacity. (HEPES = *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, Sigma Chemical Co., St. Louis, Mo.) The slight osmotic contribution made by these addition was compensated for by an appropriate reduction in NaCl, as is explained below.

Osmotic readings can be used in calculations like concentration values when mixing solutions or making media of desired osmolarity from stock solutions of known osmolarities. Thus it is a simple matter to prepare first a basal medium minus a small proportion of its usual content of NaCl and missing a certain percentage of its final volume of water. To this basal medium one can add a concentrate of an extract under study (at a volume less than the missing volume). One can then measure the osmolarity of the still incomplete medium, calculate the NaCl now needed, and finally add the necessary NaCl plus the missing volume of water. These three additions dilute the basal to its correct final volume and the other constituents (e.g., amino acids) to their desired concentrations.

Table I illustrates the preparation of two media in this fashion. The basal medium used in both examples contained all the nutrients necessary for our organ cultures, including the extra 5 mM glucose and the 20 mM HEPES. However, Basal 727 was missing 60% of its final volume of water and contained only 16 mM NaCl instead of the usual 125 mM. These two combined omissions gave a final osmolarity of 280 mosmol/kg to this basal. In example 1 the addition of the missing volume in the form of normal saline (290 mosmol/kg) diluted the nutrients to their correct concentrations and yet maintained the osmolarity in the desired range. The final value was 288 mosmol/kg, according to measurements in an osmometer (Advanced Instruments, Inc., Needham Heights, Mass.).

TABLE I

Preparation of Medium Containing a Pool Concentrate or Extract

	Components			Intermediate or final solution	
		Osmolarity	Volume	Volume	Osmolarity
		mosmol/kg	*ml*	*ml*	*mosmol/kg*
Example 1	Basal 727 (+16 mM NaCl only −60% final volume)	280	40		
	Normal saline	290	60		
				100	288
Example 2	Basal 727 (+16 mM NaCl only −60% final volume)	280	4		
	Extract of lymph node fragments eluted from CM-cellulose in 0.01 M Tris	0	4.3		
				8.3	144
	NaCl (1,250 mM)	2,350	0.68*		
	Distilled water	0	1.02‡		
				10.0	285

Abbreviations in formula: Z = osmolarity; V = volume; $_1$ = basal + extract; $_2$ = 1,250 mM NaCl solution; $_3$ = final medium.

* $(Z_1 \cdot V_1) + (Z_2 \cdot V_2) = (Z_3 \cdot V_3)$; $(144 \cdot 8.3) + (2{,}350 \cdot V_2) = (280 \cdot 10)$; $V_2 = 0.68$ ml.

‡ $10 - (8.3 + 0.68) = 1.02$ ml distilled water.

In example 2 the same basal medium was used but a portion of its missing was volume filled by a partially purified lymph node extract of negligible osmolarity (0.01 M Tris buffer). The mixture of the basal and this extract yielded a final volume of 8.3 ml with an osmolarity reading of 144 mosmol/kg. For the complete medium to have the desired osmolarity we calculated that we needed to add 0.68 ml of a concentrated NaCl solution (1,250 mM; 2,350 mosmol/kg) and 1.02 ml of distilled water. (These simple calculations are given below this example.) The final osmolarity measurement was 285 mosmol/kg.

Depiction of Data and Their Mathematical Treatment.—The antibody responses were measured by hemagglutination titration of medium changes using tanned sheep erythrocytes (Stavitsky's modification of the Boyden procedure [3]). The titrations employed intermediate dilutions—i.e., 10, 15, 20, 30, 40, 60, etc. The approximate end point was first ascertained. Then each sample was diluted in one to three steps to near its end point before preparing the final intermediate dilutions and obtaining the more precise end point. The dilution scheme used here and other details of the titration procedure are given elsewhere (4).

In this paper the data are presented mainly as graphs of differential responses, in which the ordinate indicates the average titer of media removed from a group of similarly treated cultures and the abscissa the time of the medium changes. In an experiment involving lymph nodes from one rabbit and employing four to six cultures per group, average responses of

groups clearly approximate one another during periods of identical treatment. The effects of different media were evaluated by one of two mathematical treatments. The first entailed comparing the sum of the average titers of each experimental group with that of a control group during the same period (e.g., Fig. 1). These intergroup comparisons were evaluated statistically using the two-way analysis of variance and the Dunnett multiple comparisons test (4).

A different mathematical treatment is preferable when many variables must be tested together and the number of cultures assigned each group in an experiment is limited to only three or four. The average responses of these smaller groups invariably parallel one another in their course before treatment but may not be at precisely the same level. (See Fig. 6 and note the initial mean titer of each group listed in small type at the start of each curve.) In these experiments the effects of different media were evaluated by intragroup comparisons, that is, by comparing the average response during treatment to the average response immediately before in the same set of cultures. Thus the sum of the average titers during an "assay" period (including the treatment interval and sometimes one or two succeeding medium changes) was compared with the sum of the average titers during the preceding "reference" period to give an "assay/reference" value expressed as a percent.

Appraising such percentage values in an experiment is easier when the control response is 100%, or near it. Thus an intragroup comparison may involve four to five medium changes for the reference period and three to four for the succeeding assay period, depending on the slope of the control response curve. For example, in Fig. 6 the control response (curve *a*) was still rising during the initial reference period. Therefore, five medium changes provided the reference sum of 10,985 (see numbers in small type below curve *a*), while only three medium changes yielded the assay sum of 10,666. With this combination, the assay/reference value of the control group was 97%. Intragroup comparisons (i.e., assay period titers vs. reference period titers) were evaluated statistically using the Student's t test. It should be emphasized that all comparisons made in this paper (intergroup or intragroup) rest on two sets of titers, each set derived from at least 12 and sometimes as many as 40 separately assayed samples.

Experimental I: Culture System

Effect of Osmolarity of the Medium.—Most of the following studies concern the immunosuppressive effect of new media supplemented with concentrates of used media or with extracts of lymph node cultures. Such supplements may alter substantially the osmotic pressure of the final media. And deviations from isotonicity reduce in vitro responses measured over more than a day or so. Fig. 1 illustrates the effects of osmolarity on antibody synthesis in rabbit lymph node organ cultures. Graph *A* depicts 21-day responses in several hypotonic media, while graph *B* shows the responses in several hypertonic media. Hypotonic media impaired more noticeably the later period of the 21-day responses, while hypertonic media compromised more strikingly the early period. Optimal, sustained synthesis occurred in media with osmolarities of 272 and 296 mosmol/kg, hence our preferred value of 280 mosmol/kg.

Period of Optimal Inhibition by AIM.—The inhibitory activity of mid pool concentrates was originally recognized in an experiment similar to that shown in Fig. 2, graph *A*. In preparation for this assay experiment used media from a preceding anti-BGG experiment had been collected into an early-late pool (medium changes from days 3, 6, and 18) and a mid pool (days 9, 12, and 15 medium changes). These pools were concentrated in Visking tubing (Visking

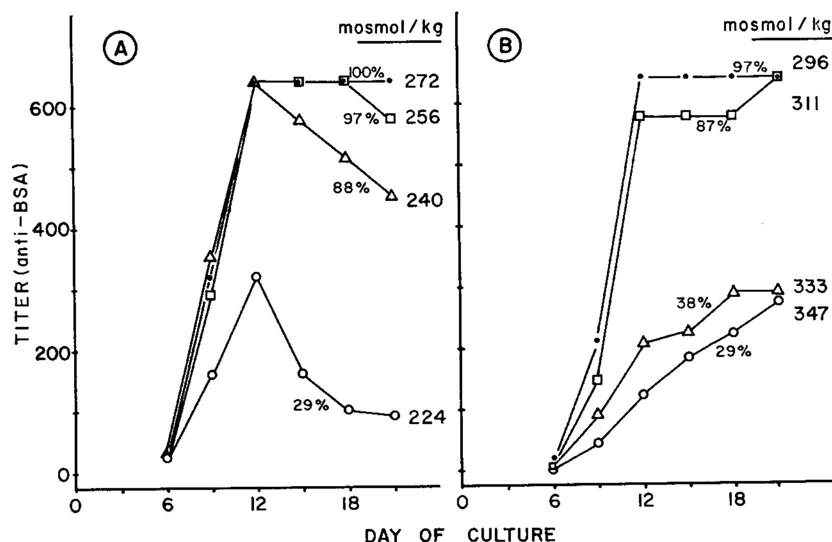

FIG. 1. The secondary antibody response of rabbit lymph node cultures in media with different osmolarities. After antigen stimulation in vitro, cultures were maintained for 21 days in serum-free media which differed only in their content of NaCl. The maximal response occurred in medium with an osmolarity of 272 mosmol/kg (125 mM NaCl). The other 21-day responses are calculated as percentages of this 100% control response. Graph *A*: anti-BSA responses in relatively hypotonic media. Graph *B*: anti-BSA responses in relatively hypertonic media.

Co., Chicago, Ill.) by sucrose-overlay[3] and then dialyzed against normal saline. When finally incorporated into new medium the concentration of the nondialyzable components was theoretically five times that in the original pools. One group of antitetanus toxoid (anti-TT) assay cultures (curve *a*) served as the untreated control. Other groups were treated with the two new media for the first 12 days, as indicated by the segmented bar at the top of graph ***A***. Taking the average response of the control group during the 21 day experiment as 100%, we note that the group treated with the early-late pool (curve *b*) gave a comparable 99% response, while the group treated with the mid pool (curve *c*) produced only a 67% response, indicative of inhibition by that pool.

Used media from another anti-BGG experiment were processed as outlined above into early-late and mid pools, which were seven times concentrated finally in the new medium. These pools were tested in other cultures of the same anti-TT assay experiment. In graph *B* of Fig. 2 we see that the treatment during days 0–6 with the early-late pool (curve *d*) permitted a 21 day response of 92%, while the corresponding mid pool (curve *e*) depressed the overall response to 83%. More striking were the effects of treatment during days 9–15, shown in

[3] The sucrose used in the concentration procedure was ribonuclease-free and obtained from Mann Research Labs., Inc., New York.

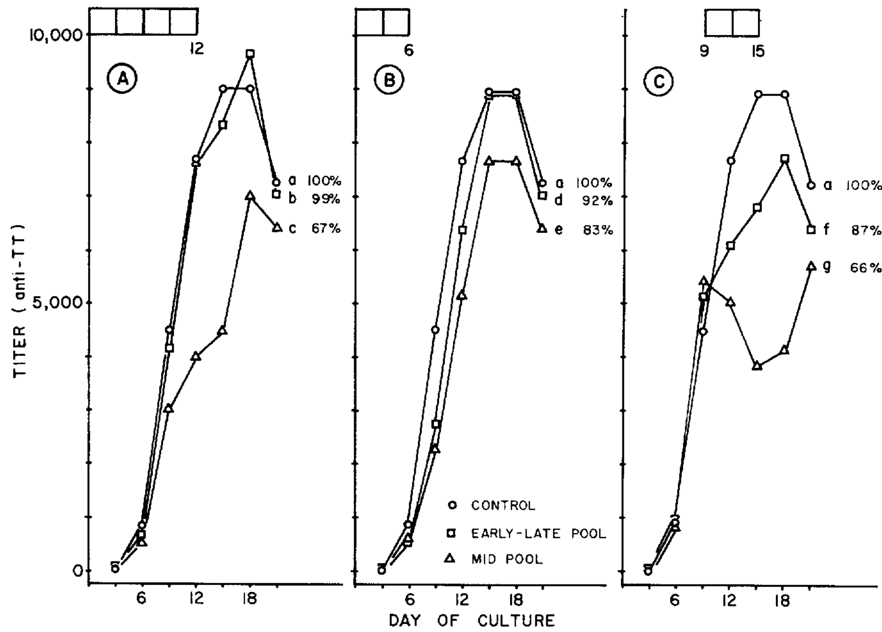

FIG. 2. The optimal period during the secondary response in vitro for the most efficient assay of AIM. The pools tested were prepared from two anti-BGG experiments (see the text). The assay system employed cultures giving an anti-TT response; their media were replaced every 3 days. One culture group received no treatment and provided the control response (100%), which is drawn in all three graphs using circles (○) (curve *a*). The responses of groups treated with media containing an early-late pool concentrate (curves *b*, *d*, and *f*) are depicted using squares (□). The responses resulting from treatment with mid pool concentrates (curves *c*, *e*, and *g*) are drawn using triangles (△). The treatment periods are indicated by the segmented bar at the top of each graph. Graph *A*: the treatment period included the first 12 days. Graph *B*: the treatment period included the first 6 days. Graph *C*: the treatment period included the 6 day period between days 9 and 15.

graph *C*. Here the early-late pool (curve *f*) permitted a 21 day response of 87%, while the corresponding mid pool (curve *g*) depressed the overall response to 66%. The introduction of the mid pool on day 9 caused a sudden reversal of the response's course, which then resumed rising after day 15, when treatment stopped. There is a suggestion of slight inhibitory activity in the early-late pool in graph *C*, curve *f*.

The two conclusions reached from this experiment were that AIM affects the productive phase of this organ culture system more conspicuously than its inductive phase and that future assays for AIM should capitalize on this observation. Thus subsequent pools were tested in assay cultures during the early productive phase (days 7–14). Also these assay responses were followed at shorter intervals in order to provide a larger number of titrations and hence a greater overall statistical validity to the observations.

Effect of Arrested Mitotic Activity During the Productive Phase.—Previous histological examinations of rabbit lymph node fragments undergoing a secondary response induced in vitro had revealed abundant mitotic figures during the first 6–8 days, i.e., during the inductive phase in these cultures. Numerous mitoses were also seen during the productive phase (after day 8 or so) and even when the response was waning. We had assumed that most of the latter mitotic activity involved the proliferation of fibroblasts and other cells not directly involved in antibody synthesis. Nevertheless, as will be discussed later, the question arose whether or not this mitotic activity might influence the productive phase response in ways not considered before and might relate to AIM in some manner.

To investigate this question we employed hydroxyurea, a mitotic inhibitor previously used in spleen cultures by Jaroslow and Ortiz-Ortiz (5). The average responses depicted in graph *A* of Fig. 3 were derived from anti-TT cultures treated with 0.5 mM hydroxyurea commencing on day 4, 6, or 8. These media were replaced daily. Curve *a* represents the untreated control cultures whose 14 day response was 100%. When treatment was commenced on day 4, the ensuing response was almost completely suppressed, as seen with curve *b*. The same medium started on day 6 in another set of cultures depressed their mean response to 19%. But treatment begun on day 8 (curve *d*) affected the response only belatedly and permitted an overall response of 88%. Thus the later the addition of hydroxyurea, the less effective it is in depressing the secondary response. Presumably, by day 8 most of the antibody-producing cells are "end" cells, incapable of further replication and thus insensitive to the drug.

Graphs *B* and *C* of Fig. 3 represent a second experiment in which 0.5 mM hydroxyurea was added to cultures for only a 24 h period at different times during the response. Curve *e* is drawn in both graphs and is the same untreated control response (100%). Treatment during day 4/5 depressed the response to 59%, as shown in curve *f*. However, treatment on day 6/7 permitted a 107% response in the cultures represented by curve *g*. Thus no appreciable number of antibody-producing cells was undergoing cell division at this time.

Graph *C* presents the curious result that still later pulses of hydroxyurea invariably provoke enhancement of the response compared with the untreated control (curve *e*). Curves *h*, *i*, and *j* depict the effect of 0.5 mM hydroxyurea being added for 24 h on days 8/9, 10/11, and 12/13, respectively. The average responses of these culture groups were all greater than the control subsequent to their 24 h treatment. The implication of this observation for AIM will be discussed later.

Experimental II: Chemical Properties of AIM

Molecular Size.—Our earlier studies (1) indicated that AIM has a mol wt in excess of 10,000, since the inhibitory activity of mid pools is retained inside Visking cellophane tubing during concentration by sucrose-overlay and subse-

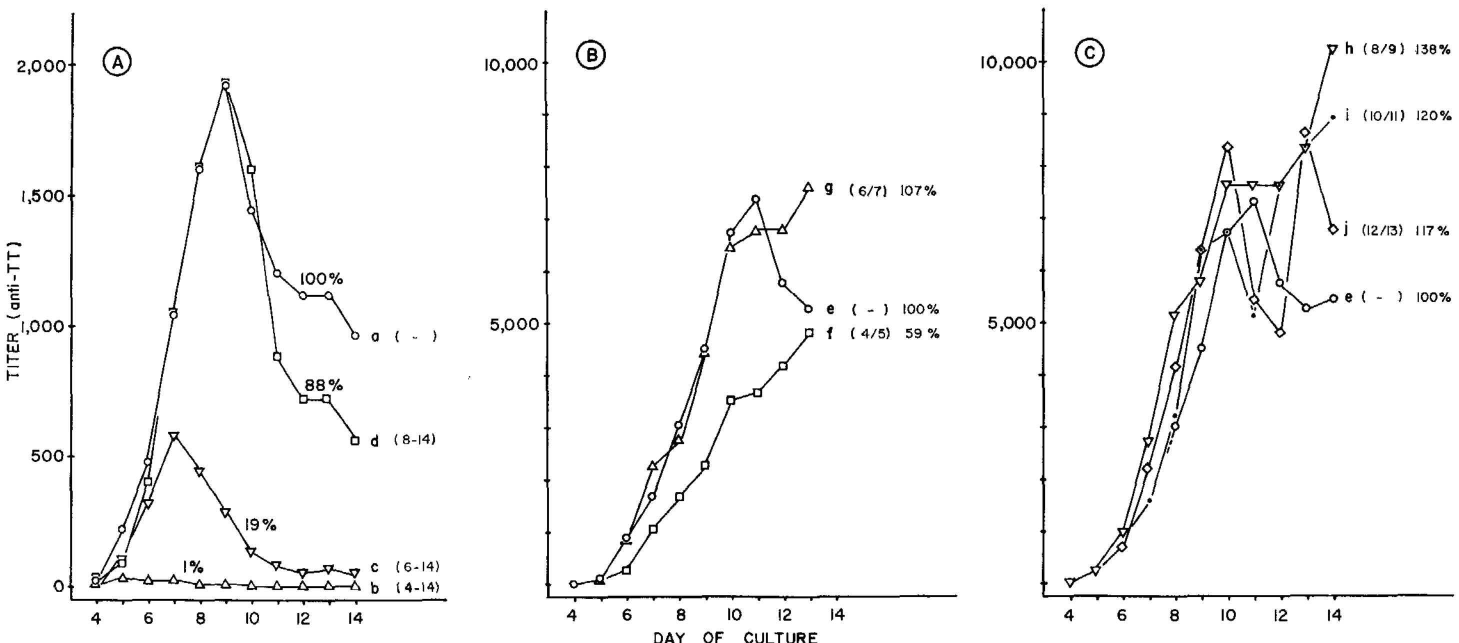

FIG. 3. The effect of hydroxyurea on the secondary antibody response in vitro. Two anti-TT experiments are summarized in this figure. The media of both were replaced daily. The period of hydroxyurea treatment in days is given by the bracketed numbers drawn beside each curve. Graph *A*: the responses drawn here represent cultures from the first anti-TT experiment whose untreated (–) control response is represented by curve *a* (100%). Medium containing 0.5 mM hydroxyurea was commenced at different times in three experimental groups and continued in each through day 14. For example, the treatment period of curve *b* was day 4 through day 14, which is designated as (4–14). Graphs *B* and *C*: the responses drawn here represent the second anti-TT experiment whose untreated (–) control response is depicted by curve *e* (100%) in both graphs. 1-day pulses of medium with 0.5 mM hydroxyurea were used in this experiment. The treatment period is given by the bracketed figure—e.g., (4/5) for curve *f*. The responses of those experimental groups treated on day 8 or later are assembled in graph *C*. These responses are recorded through day 14 and those of graph *B* through day 13.

quent dialysis against saline (6). To determine the upper size limit of AIM we employed two ultrafiltration membranes with different retentivities (7).

Media removed from an anti-BSA experiment were pooled and frozen for storage each 3 days through day 18. Later these 3-day pools were thawed and combined into an early-late pool (days 3, 6, and 18) and a mid pool (days 9, 12, and 15). Each pool was first centrifuged and then sterilized through a Millipore filter (Millipore Corp., Bedford, Mass.) (HA 0.45 μm), concentrated by sucrose-overlay, and dialyzed against saline. Each starting pool volume was reduced from 135 ml to 15 ml. The two concentrates were filtered through a Diaflo XM-50 ultramembrane (Amicon Corp., Lexington, Mass.) (exclusion limit of 50,000), followed by two 5 ml normal saline rinses. Each final filtration was stopped with 3–4 ml of retentate still remaining. These retentates were dialyzed against normal saline and then incorporated into new medium. The two ultrafiltrates were concentrated on a Diaflo UM-2 ultramembrane (exclusion limit of 1,000–2,000) to a volume of 3-4 ml, dialyzed against normal saline, and incorporated into new medium.

The four new media were tested on day 13–14 in antidiphtheria toxoid (anti-DT) cultures whose responses are drawn in Fig. 4. Neither of the two fractions from the early-late pool (graphs *A* and *B*) nor the XM-50 retentate from the mid pool (graph *C*) were inhibitory. Only the XM-50 ultrafiltrate of the mid pool (graph *D*) depressed the assay response. Thus AIM has a mol wt in the range of 10,000–50,000 daltons.

Absorption Chromatography.—Initial attempts to isolate AIM by column chromatography were unrewarding, possibly because the additional concentration and dialysis of the eluates may have reduced the inhibitory activity. To minimize such manipulations we employed a batch absorption technique in which [*O*-(carboxymethyl)cellulose] CM- or [*O*-(diethylaminoethyl)cellulose] DEAE-cellulose was centrifuged dry in a modified Hemmings' filter apparatus.

This assembly (Colab Laboratories, Inc., Glenwood, Ill.) consists of two 5 ml screw-capped glass bottles whose open ends are joined through a central metal connector in which is a sterilizing filter, supporting screen, and washers. The apparatus is designed to sterilize small volumes of fluids by filtration under centrifugal force. For our particular use we sealed the air release in the side of the metal connector with solder, replaced the sterilizing filter with a sintered plastic screen to retain cellulose, and replaced one glass bottle with a 10 ml screw-capped plastic centrifuge tube. The bottom of this tube was cut off so that it functioned as a filter holder when inverted, while the opposite glass bottle served as the receptacle. The assembled, modified apparatus has a height under 110 mm and thus swings freely in a 50 ml centrifuge cup.

CM-cellulose and DEAE-cellulose were prepared according to the method of Peterson and Sober (8) and finally suspended in appropriate buffers—0.01 M Tris at pH 5.3 and 7.0, respectively. A 10 ml thick slurry of each (ca. 0.5 g dry weight) was added to separate filter assemblies and centrifuged to relative dryness for 5 min at 1,000–1,500 rpm. Each batch of packed cellulose was resuspended and washed in its appropriate starting buffer three times before receiving 5 ml of a concentrated anti-BGG mid pool, which had previously been dialyzed against the appropriate starting buffer. The slurry was repeatedly stirred for 5 min before centrifugation and recovery of the first eluate (0.01 M). Each batch of packed cellulose then received 5 ml of 0.5 M Tris at pH 7.0 (for CM-cellulose) or pH 5.3 (for DEAE-cellulose). After further stirring of each slurry for 5 min we obtained the second set of eluates (0.5 M) upon centrifugation. (A second elution with 0.5 M buffer yielded only 11–28% of the 280 nm absorb-

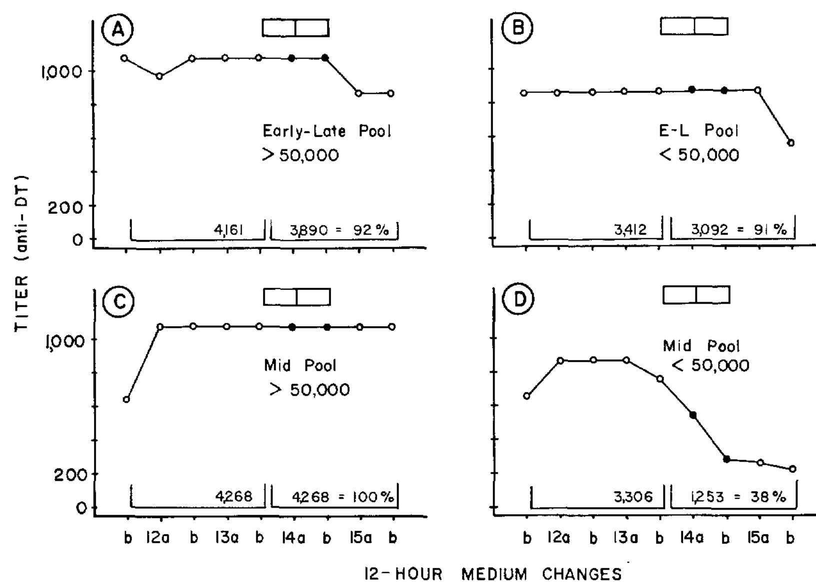

FIG. 4. The approximate molecular size of AIM. The assay system employed anti-DT cultures whose media were replaced every 12 h during the period under study (days 8–16). The early-late pool and mid pool of an anti-BSA experiment were processed in parallel fashion (see the text). Each yielded a retentate with a molecular size greater than 50,000 daltons (designated by >50,000 in graphs *A* and *C*) and a filtrate containing material of the range 10,000–50,000 (designated by <50,000 in graphs *B* and *D*). In each graph the reference period included four medium changes (12 *a*–13 *b*). The treatment period of 24 h included medium changes 14 *a* and *b* and is represented by the segmented bar at the top of each graph. The assay period included four medium changes (14 *a*–15 *b*). At the bottom of each graph are given the sum of the average response for the reference period and the sum for the assay period. The assay/reference value is the percentage figure given there (see Material and Methods).

ing material eluted by the previous 0.5 M buffer step.) The two main sets of eluates were dialyzed for 3 h against several changes of normal saline, after which time the osmotic readings of the eluates were around 280 mosmol/kg. These four saline-dialyzed eluates were titrated for their anti-BGG levels and then incorporated into new medium for testing in a heterologous assay culture system.

The relative amount of 280 nm absorbable material in each eluate added to each of the four new media was obtained by multiplying (*a*) the OD_{280} reading of that eluate by (*b*) the volume of eluate incorporated per milliliter of new medium. This concentration is expressed here as OD_{280} units per ml medium or ODU_{280}/ml.

Graph *A* of Fig. 5 depicts the average responses of two sets of control cultures during the period under study when the media were replaced every 8 h. The parallel, relatively steady responses of these two control groups contrast with the inhibition produced by the media containing the eluates.

Graphs *B* and *C* concern the eluates of the CM-cellulose. Media containing the 0.01 M, pH 5.3 and the 0.5 M, pH 7 eluates were added at three changes

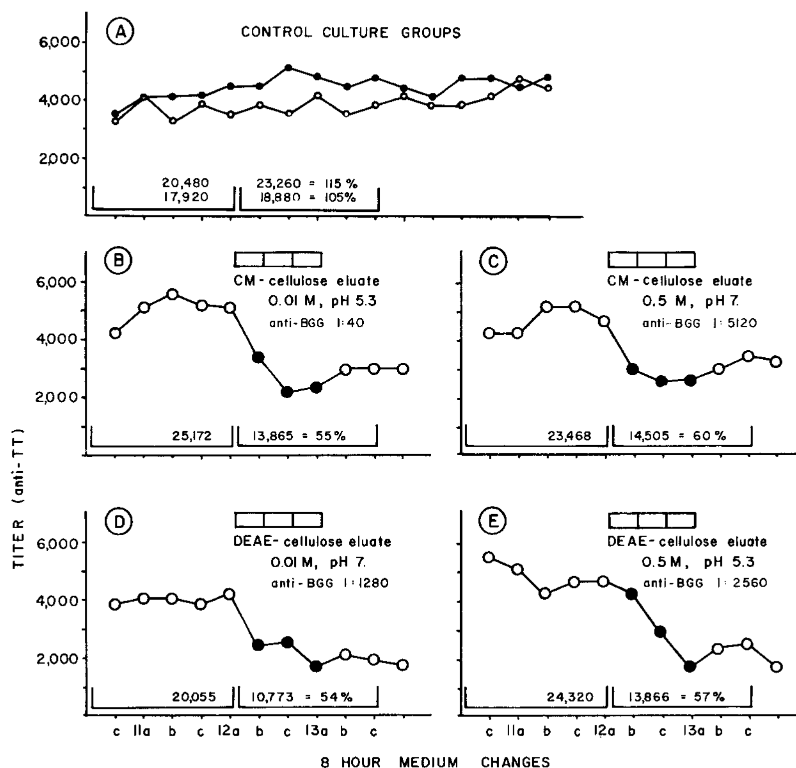

FIG. 5. Behavior of AIM during batch absorption chromatography. The assay system involved anti-TT cultures, whose media were replaced every 8 h during the period under study (days 8–16). The material assayed was a concentrated anti-BGG mid pool, which was divided into two samples for dialysis against the different starting buffers (see the text). The reference period included the five medium changes 10 *c*–12 *a*. The treatment period encompassed three medium changes (12 *b*–13 *a*), as indicated by the segmented bar above each experimental group response. The assay period included the five medium changes 12 *b*–13 *c* The sums of the average responses of each group during the reference and assay period are recorded along with their assay/reference percentage values.

on day 12, as indicated by the segmented bar across the top of these graphs. Both eluates produced prompt inhibition. The titer of the original concentrated anti-BGG mid pool was 5,120. The efficiency of the CM-cellulose batch absorption for antibody is evident from the 0.01 M eluate having a titer of only 40, while the 0.5 M eluate had a titer of 5,120. The first eluate, which contained only 0.11 ODU_{280}/ml from the mid pool, was as inhibitory as the second eluate, which contained 0.42 ODU_{280}/ml. Since the CM-cellulose was not rinsed with the starting buffer before adding the 0.5 M buffer, the inhibitory activity found in the second elution may represent AIM remaining in the cellulose but not absorbed to it. However, the useful finding here is that AIM can readily be separated from the antibody of these cultures by use of CM-cellulose.

Graphs *D* and *E* concern eluates of DEAE-cellulose. As above, media containing the two DEAE eluates (0.01 M, pH 7 and 0.5 M, pH 5.3) were added during the period designated by the segmented bar atop each of these graphs. Again both eluates were inhibitory. The anti-BGG titers were similar in the two eluates. (Practically all antibody present here is 7S, since the pools were obtained during the height of the secondary response in vitro [9]). The ODU_{280}/ml values for these two media were 0.06 and 0.46, respectively, and thus do not parallel their relative inhibitory activities. The background of other protein may be responsible but also significant may be the 260/280 absorption ratios of the dialyzed pools, which ranged from 1.2 to 1.6, indicating the presence of significant amounts of nucleic acids. The implication of this latter observation was realized only after this experiment and will be discussed below.

Adherence of AIM to UM-2 Diaflo Membranes.—In our initial studies with AIM its irregular recovery from mid pools was generally correlated with the magnitude of the secondary responses associated with them. However, sometimes activity was missing from mid pools of cultures which had responded well. This suggested the possible lability of AIM or its loss during isolation or fractionation procedures. An example of the latter appears to be the adherence of AIM to certain Diaflo membranes.

Mid pools from one BGG and two BSA experiments were combined and reduced in volume from 120 ml to 6 ml by concentration on a UM-2 Diaflo ultramembrane. The UM-2 membrane was then rinsed with several changes of normal saline which were pooled together. The original UM-2 retentate and the combined rinses were dialyzed against normal saline and incorporated into new media after UV absorption readings were taken. The two new media were examined in an anti-TT assay system.

Fig. 6 depicts these results. The untreated control response represented by curve *a* had an assay/reference value of 97%. The UM-2 retentate was present in medium at a concentration of 0.13 ODU_{260}/ml (also 0.13 ODU_{280}/ml) and yielded an assay/reference value of 78% (curve *b*). The pooled saline rinse of the UM-2 membrane was tested at a concentration of 0.10 ODU_{260}/ml (or 0.08 ODU_{280}/ml); it depressed the assay/reference value to 51% (curve *c*). The greater inhibitory activity of the UM-2 rinse suggests that AIM absorbs to this particular ultramembrane. The implications of this phenomenon for the chemical nature of AIM will be discussed later.

Stability of AIM to Acid Dialysis.—An early consideration in this study was that AIM might be interferon. This was somewhat dispelled by the observation that high titers of interferon in serum of Sindbis-infected rabbits failed to inhibit the secondary response in vitro (1). But before this finding we had tested the stability of AIM to acid dialysis, a treatment few proteins besides interferon can withstand.

An active rinse of a UM-2 membrane which had previously been used to concentrate a mid pool was divided into two samples. One was dialyzed for 20 h at 5°C against several changes

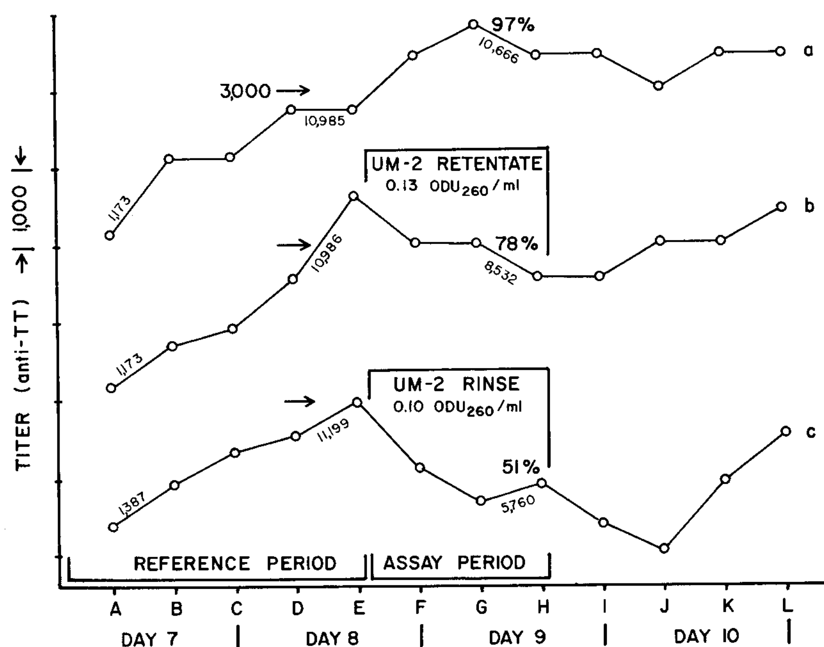

FIG. 6. Recovery of AIM in rinses of a Diaflo UM-2 ultramembrane previously used to concentrate mid pools. The assay system involved anti-TT cultures whose media were replaced every 8 h during the period under study (day 6–11). The titrated medium changes are designated by the letters A–L. The average responses of the three groups of this experiment have been staggered one above another along the ordinate to avoid overlapping curves. But each response had a starting mean titer (medium change *A*) of slightly greater than 1,000. On the ordinate the interval between each mark is 1,000 dilution units. The 3,000 titer level for each response is indicated by an arrow beside each of the three curves. Curve *a* represents the response of the untreated control group. Mid pools from heterologous responding cultures were concentrated on a Diaflo UM-2 ultramembrane. The relative inhibition produced by the UM-2 retentate and the pooled rinses of this membrane are represented by curves *b* and *c*, respectively. (See the text.) The reference period on days 7 and 8 included five medium changes (A–E). The treatment and assay periods on days 8 and 9 both included three medium changes (F–H). The reference and assay sums of each group are recorded in small type below its response curve. The assay/reference percentage value is given above each curve at the end of the assay period.

of normal saline maintained at pH 2 and then dialyzed against several changes of normal saline-buffered at pH 7.2. The control sample was dialyzed during the entire 20 h period against the latter buffer with the same number of changes.

Graph *A* of Fig. 7 represents the untreated control response, which had an assay/reference value of 108%. Graph *B* shows the inhibition evoked by medium containing the mid pool dialyzed only against the pH buffer; here the assay/reference value was depressed to 45%. Graph *B* depicts the response permitted by AIM dialyzed against the pH 2 saline solution; the corresponding assay/

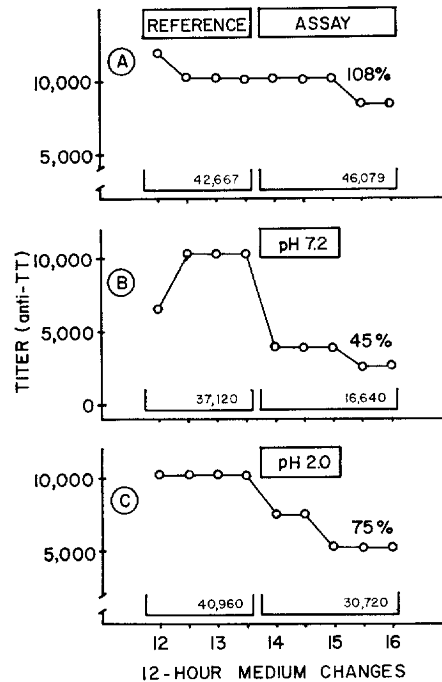

FIG. 7. The effect of acid dialysis on AIM. The assay system involved anti-TT cultures whose media were replaced every 12 h during the period of study (days 9–17). The reference, treatment, and assay periods are indicated by the rectangles at the top of the graphs. The sums of the reference and assay responses and the assay/reference percentage value for each group are recorded in each graph. Graph *A*: response of the untreated control group. Graph *B*: response of an experimental group treated with a mid pool concentrate which had been dialyzed against normal saline at pH 7.2. Graph *C*: response of an experimental group treated with a mid pool concentrate which had been dialyzed against normal saline maintained at pH 2.

reference value was 75%, which indicates inhibitory activity somewhat less than that contained by the mid pool sample not acidified. The partial resistance of AIM to acid dialysis will be considered again later.

Enzyme Digestion.—Using pronase coupled to Cellex-PAB (10) and also commercially obtained Enzite-papain (Miles-Servac Ltd., Maidenhead, Berks., England), we sought to confirm the presumed protein character of AIM. The enzymatic potency of our pronase-PAB preparation was verified by its ability to reduce the anti-TT titer in a medium from 640 to 80 after a 3 h digestion and to 20 after 13 h. In two experiments the inhibitory activity of mid pools was not destroyed by these proteolytic enzyme preparations.

However, treatment of active pools with ribonuclease abolished inhibitory activity in several experiments and reduced it significantly in others. The examples presented here illustrate two methods of digestion.

In this first example an active mid pool concentrate was divided into two 3 ml samples. One was treated with 12 mg of Enzite-ribonuclease (1.0 μmol CCP hydrolyzed/min per mg) on a magnetic stirrer for 2 h at room temperature and pH 6.5 and then separated from the enzyme by centrifugation. The other sample was treated in a parallel fashion except no enzyme was added. Both samples were incorporated into new medium for testing in a heterologous assay system.

These results are shown in Fig. 8, which has a format exactly like that of Fig 6. The reference period included the five medium changes designated A–E and

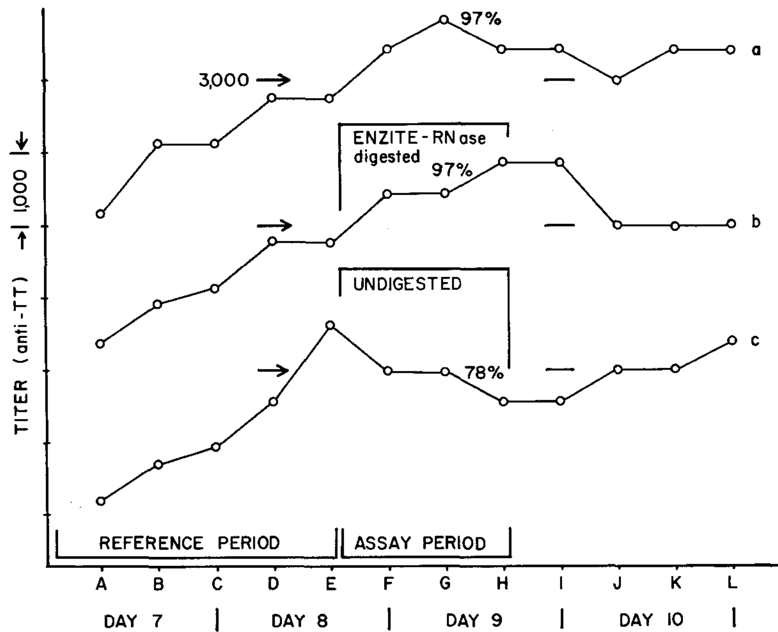

FIG. 8. Loss of inhibitory activity after digestion of AIM with Enzite-ribonuclease. The format of this figure is like that of Fig. 6. Shown here are the average responses of an untreated control group (curve *a*), of a group treated with a mid pool sample previously digested with Enzite-ribonuclease (curve *b*), and of a group treated with the undigested midpool sample (curve *c*).

the treatment (and assay) period those changes designated F–H. The assay/reference value for the untreated control group (curve *a*) was 97%. The group treated with the RNase digested mid pool had an assay/reference value of 97% also (curve *b*). However, the undigested mid pool sample reduced the assay/reference value to 78% in a third group of cultures (curve *c*). The difference between the assay period of curve *c* and the corresponding periods of curves *a* or *b* is statistically significant at $p < 0.01$, according to the Dunnett multiple comparisons test.

Fig. 9 similarly illustrates the loss of inhibitory activity of another mid pool

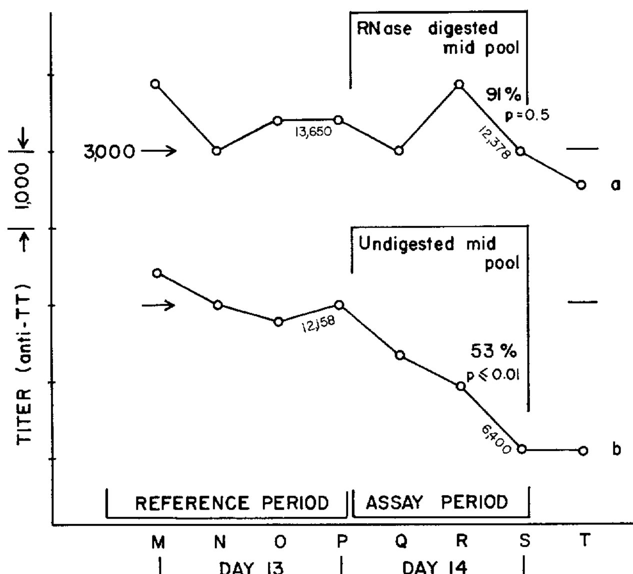

FIG. 9. The loss of inhibitory activity after digestion of AIM with ribonuclease. The assay system involved anti-TT cultures whose media were replaced every 8 h during the period of study (days 8–15). The medium changes pertinent to this experiment are designated M–T. (days 13–15). As in the preceding figure, the response curves here are staggered one above the other with the 3,000 titer level for each response being noted with an arrow. Curve *a* represents the average response of cultures treated with a mid pool sample previously digested with ribonuclease. Curbe *b* depicts the inhibitory effect of an undigested mid pool sample.

sample treated with 100 μg/ml ribonuclease-A (bovine pancreas, Sigma Chemical Co.) for 80 min at 37°C, pH 7. After this digestion the sample was separated from the soluble enzyme by passage through a G-50 Sephadex column. A control sample not digested was run through an identical G-50 column and parallel fractions collected. Based on 260 nm absorption readings, an equivalent amount of the eluate from each column was incorporated into two new media. These were tested during the three medium changes (Q-S) on day 14 of an anti-TT assay experiment. Medium containing the RNase digested sample gave an assay/reference value of 91% in cultures represented by curve *a*. The nondigested sample, however, depressed the assay/reference value to 53% in another set of cultures (curve *b*). According to analysis by the Student's *t* test, this depression is significant at $p < 0.01$, while the 91% value of curve *a* does not represent a significant depression ($p = 0.5$).

Extraction of AIM from Stimulated Lymph Node Fragments in Culture.—The accumulation of used medium was a time-consuming aspect of this investigation. Therefore, we sought to recover inhibitory activity directly from the cultured lymph node fragments early in the productive phase. For this purpose nine cultures of an anti-TT assay experiment were sacrificed on day 8. These

were termed "young" fragments, for at the same time three older experiments were terminated and their cultures yielded a combined extract of "old" fragments. The details of the preparation of these two extracts differed slightly and are outlined in Table II.

Because both extracts contained high titers of anti-TT and because the assay

TABLE II

Extraction of AIM from Cultured Lymph Node Fragments (See Fig. 10)

Details of procedure	"Young" fragments	"Old" fragments
Source of sample	180 fragments (fgts), anti-TT, day 8 (autochthonous for the assay cultures)	400 fgts, anti-TT, day 17 300 fgts, anti-BSA, day 24 200 fgts, anti-BGG, day 24
Extraction solution	9 ml of 2 day old medium containing free-floating cells from the same fgts	65 ml distilled water
Extraction method	Freezing in alcohol-CO_2 bath and thawing once	Freezing in alcohol-CO_2 bath and thawing 1x
Additional rinse of extracted fragments	6 ml distilled water added to centrifuged debris	None
Sterilization by Millipore filtration (HA 0.45 μm)	Yes	No
Anti-TT titer at this stage	ND	10,240
Concentration on UM-2 membrane	15 ml → 6 ml	65 ml → 6 ml
Anti-TT titer at this stage	10,240	81,000
Batch absorption by CM-cellulose	Yes	Yes
Anti-TT in first eluate	80	<10
$OD_{260/280}$ of first eluate*	1.74	1.26
ODU_{260} of eluate/ml medium*	0.052	0.056
ODU_{280} of eluate/ml medium*	0.088	0.045

* Corrected for turbidity by subtracting OD_{315} reading.

culture involved the same response, this antibody was removed by batch absorption onto CM-cellulose, as described previously. The anti-TT titer of 80 in the eluate of the young fragment extract was negligible compared with the range of titers (2,000–3,000) in the assay cultures at the time of treatment. The 260/280 absorption ratios for the two final preparations were 1.74 and 1.26, indicating an appreciable content of nucleic acids. (The $OD_{260/280}$ ratio of serum is around 0.65 and that of pure RNA or DNA is 2.0.) The two new media contained approximately equal amounts of the two extracts, based on the ODU_{260}/ml values for the eluates (0.052 vs. 0.056).

Fig. 10 illustrates the potency of these extracts. Curve *a* is the untreated control response, which had an assay/reference value of 97%. (The same control response also pertains and is depicted in Fig. 8.) Medium containing the extract of the young autochthonous fragments reduced the assay/reference value for the cultures of curve *b* to 70%. The extract of the old fragments depressed this value for other cultures (curve *c*) to 55%.

DISCUSSION

The study of AIM has proved difficult because of the assay system, which is complex and highly consumptive of the isolated material. The isolation itself

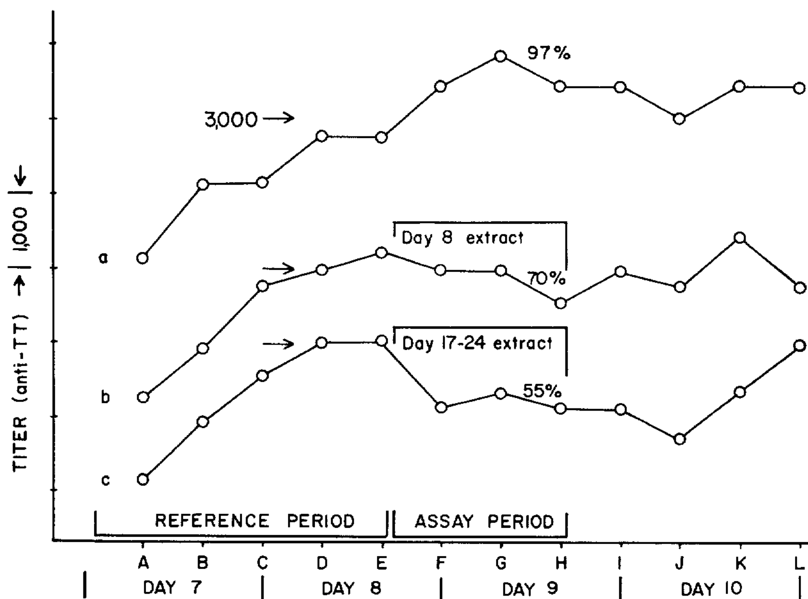

FIG. 10. Extraction of AIM from cultured lymph node fragments. The format of this figure is like that of Fig. 6. Curve *a* is the control response of the untreated culture group. Curve *b* derives from the experimental group treated with an extract of 8-day old autochthonous lymph node fragments. Curve *c* represents the response of the experimental group treated with an extract of heterologous responding fragments sacrificed after 17–24 days in culture. (See the text and Table II.)

was initially complicated by unexplained losses of activity and by our uncertainty about optimal conditions for the production and collection of the inhibitory material. The resolution of these latter difficulties led to several new considerations about the regulation of the immune response. The following discussion first compares AIM with other well-described immunosuppressive factors and then deals with some implications stemming from the RNA character of AIM.

AIM and Other Immunoregulatory Factors.—The effectiveness of AIM during the productive phase distinguishes it from several other naturally occurring factors which suppress the immune response. For example, the factor(s) studied by Mowbray's group—the α_2-glycoprotein isolated from serum (11) and the RNase complexes (12)—suppress hemolysin responses in mice maximally when injected intravenously 10–20 h before antigen stimulation. A comparable factor examined in Cooperband's laboratory (13) was reported to suppress primary and secondary plaque responses in mice injected intravenously 16–20 h before primary or secondary challenge with sheep erythrocyte. These factors have little or no effect when injected after antigen stimulation and apparently depress the response by a mechanism different from that of AIM.

Our hydroxyurea experiments described above indicated that the antibody-producing cells in rabbit lymph node organ cultures are undergoing little mitotic activity during the productive phase, when AIM is best assayed. Therefore, we doubt that AIM is related to various lymphoid-derived factors with pronounced antimitotic activity. For example, an extract which inhibits mitoses but is not cytotoxic when added to human peripheral leukocytes stimulated with phytohemagglutinin (PHA) was obtained by Moorehead et al. (14) from porcine lymph nodes. Similar substances have been isolated from lymphoid tissues of other species by Garcia-Giralt (15) and Houck et al. (16). A labile, nondialyzable factor which inhibits the proliferation of HeLa cells without affecting their viability has been found in cultures of PHA-stimulated human leukocytes by Green et al. (17). Since AIM inhibits antibody synthesis in end cells, it must function by a mechanism other than inhibiting DNA synthesis.

Lymphotoxins and other soluble inhibitors released from lymphocytes stimulated with PHA, various antigens, or other cells can be distinguished from AIM by their nonspecific elaboration, their general cytotoxicity, their larger size, etc. (18). In addition, such products are released within hours after stimulation (19, 20). This early release contrasts with the appearance of AIM, which is not found in our cultures until a week or more after antigenic stimulation.

One immunosuppressive factor recently reported bears some resemblance to AIM. Veit and Michael (21) tested the sera of nonimmunized mice and of mice injected with horse erythrocytes 4 days before. Normal syngeneic spleen cells were incubated for 4 h in such sera along with one of two antigens (sheep red blood cells or *Escherichia coli* 0127 lipopolysaccharide) and then transferred intravenously to irradiated recipient mice along with an immunizing dose of the corresponding antigen. 6 days later the spleens of the recipient mice were examined

by the Jerne assay method. The response to the sheep erythrocytes (thymus dependent) was suppressed by the incubation step, but the response to the lipopolysaccharide (thymus independent) was not affected. The incubation with normal serum suppressed the sheep cell response 20%, while the immune serum (at the same dilution) produced a 71% suppression. The authors postulate that this serum factor serves as a "continually acting control system regulating the immune response." One difficulty in relating this factor to AIM is the latter's sensitivity to ribonuclease. The high levels of such enzymatic activity in serum (22) militate against recovery of AIM from this source (see discussion below).

The Reversible Action of AIM.—An informative property of any inhibitory material is whether its effect is reversible. We have noted frequent instances of in vitro responses first being depressed by AIM but then rising after addition of new medium devoid of the material (see Figs./curve: 2/*g*, 6/*c*, 8/*c*, and 10/*c*). Such posttreatment rises could result from a proliferation of antibody-producing cells afterwards. However, our hydroxyurea studies suggest that no such proliferation occurs during the productive phase in this system and support the view that the resumption in synthesis is due to a reversible effect of AIM on the existing population of antibody-producing cells.

The RNA Character of AIM.—Four other observations are consistent with our ribonuclease experiments and the conclusion that AIM has an essential RNA component. (*a*) During batch fractionation with CM-cellulose a high level of AIM activity was recovered in the initial eluate (0.01 M, pH 5.3). Under these conditions nucleic acids are negatively charged and not absorbed to cellulose.

(*b*) The apparent adsorption of AIM onto Diaflo UM-2 membranes was another unexpected finding compatible with the RNA character of AIM. More precisely, this observation suggests that AIM is strongly charged, according to the following line of reasoning. UM-2 filters are noncellulosic polymeric membranes formed by the interaction of two synthetic polymers of opposite charge (polyvinyl-benzyl-trimethyl ammonium chloride and polystyrene sulfonate). The stoichiometric proportions normally used yield a net neutral charge to the membrane but do leave "some ionic sites" (see Amicon Corp.'s publication no. 426, p. 6). Such localized sites may account for the accumulation of dialyzable molecules (electrolytes) in the retentate of this type membrane—a phenomenon evident from the high osmolarity of media concentrated by this means. (For example, pools with a starting osmolarity of 280 mosmol/kg yield concentrates with osmotic readings well over 400 mosmol/kg, which can readily be reduced by dialysis against normal saline. Parenthetically, such undialyzed retentates would be inhibitory to cultures merely from hyperosmotic effects or from toxic levels of certain ions.) The localized ionic charges on UM-2 membranes may directly, or indirectly, cause adsorption of AIM. In retrospect, a 4 M salt solution might have been a more effective rinse than the normal saline we used, since this higher molarity would provide more counter ions to break the postulated AIM membrane bonds.

(*c*) Yet another observation consistent with the nucleic acid character of AIM is its relative stability to acid dialysis. (*d*) Finally, we believe that AIM is lost under certain biological conditions associated with significant RNase activity. The latter idea will be explored in the following section.

Loss of Inhibitory Activity.—Initial studies on a biological factor are sometimes clouded by the possibility of its degradation during isolation or storage. This concern is important when measuring levels of activity under different conditions or from different sources. Thus a valid question is whether the level measured represents the amount actually produced or whether it reflects also significant destruction by tissue enzymes or loss by some other means. Two examples will illustrate this problem in the case of AIM.

In experiments not described extensively here we attempted to extract inhibitory activity from lymph nodes of a BGG-primed rabbit before and 6 days after a secondary injection of BGG. The preinjection nodes were removed under ether anesthesia before stimulation and stored frozen until the animal was sacrificed 6 days later. Then both sets of nodes were homogenized and centrifuged to yield supernatant extracts for incorporation into new medium and testing in anti-TT assay cultures. No inhibitory activity was found in either node preparation, although other cultures in the assay were inhibited by an extract of autochthonous 9-day old fragments. Data reviewed below suggest that lymph nodes freshly removed from a rabbit have higher RNase levels than lymph node fragments after culture for several weeks in serum-free medium.

The second example concerns the inhibitory activity recovered from lymph node organ cultures of different ages. Fig. 10 illustrates the finding that old cultures (days 17–24) yielded approximately twice as much inhibitory activity as did young cultures (day 8) when equivalent amounts of each extract were included in the two new media (i.e., "equivalent" in terms of ODU_{260}/ml of extractable material). Table II gives the protocol followed for both sets of fragments and points out differences in the starting samples and in their processing which might account for the two levels of activity. However, two other possible explanations are pertinent to this discussion: (*a*) that young cultures have higher levels of degradative enzymes and thus yield lower levels of AIM upon extraction and (*b*) that old cultures have "accumulated" more AIM through the proliferation of inhibitor-containing cells. The first idea will be discussed immediately below. The second idea is implicit in our studies on the cellular source of AIM described in the succeeding paper of this series.[2]

RNase Activity in Lymphoid Organs In Situ and in Lymph Node Cultures.—Reports in the literature on ribonuclease activity in normal vs. stimulated lymphoid tissue have yielded different findings for spleen and lymph nodes. Chakrabarty and Friedman (23) measured RNase activity at pH 5.8 in mice immunized with sheep erythrocytes. The spleens showed a fall in activity during the first 3 days after immunization and then a return to the normal level. However, the mice lymph node homogenates exhibited a threefold rise which peaked 2 and 6 days after immunization and which on the 8th day was still double that

of the nonimmunized control value. Maor and Witz (24) measured alkaline ribonuclease levels in lymph nodes of rats injected with sheep erythrocytes. They observed a threefold rise, which peaked 2 days after injection and returned to normal by the 5th day.

The above reports attest to the variation in levels of certain ribonucleases in lymphoid organs. But mammalian tissues contain at least seven distinct ribonucleases having different substrate specificities and requiring different assay conditions (25). At present it is not known which of these enzymes or which conditions of assay should be considered in studies on the degradation of AIM. Therefore, the general avoidance of ribonucleases would seem appropriate. One such avoidable source of these enzymes is serum.

Using the method of Ambellan and Hollander (26), Hansbrough[4] found that medium containing 20% calf serum has RNase activity (pH 5.5) equivalent to 250 μg/ml bovine pancreatic ribonuclease. In marked contrast, our serum-free medium contains only 1 μg/ml RNase activity after incubation with stimulated lymph node fragments from day 12 to 15. It is reasonable to suppose that after repeated rinsing and prolonged cultivation, these fragments lose most traces of serum and are greatly depleted of the ribonucleases normally present in the lymph node *in situ*. This may account for the high activity of AIM found in 3–4-wk old cultured fragments, for the lower activity extracted from 8-day old fragments, and for the absence of inhibitory activity in extracts of fresh lymph nodes. Therefore, the serum-free medium employed in these studies may have been crucial for the isolation of AIM from used medium and from cultured fragments. Also it may be difficult to duplicate these studies on AIM in other culture systems which require serum in their medium.

SUMMARY

A material inhibiting antibody synthesis in vitro is produced during the productive phase by rabbit lymph node organ cultures undergoing a secondary response. This antibody inhibitory material (AIM) has been isolated from serum-free medium taken from the cultures and also extracted from lymph node fragments as late as their 4th wk in vitro.

AIM inhibits most strikingly the early productive phase of the secondary response in vitro (i.e, during the 2nd wk). AIM isolated from cultures undergoing a given immune response inhibits the same as well as different responses, thus indicating an immunologically nonspecific effect.

Ultrafiltration and related studies reveal that the molecular size of AIM is 10,000–50,000 daltons and that it is not antibody. AIM can readily be separated from 7S globulin by use of CM-cellulose. The inhibitory activity of AIM is lost by digestion with ribonuclease. Thus the avoidance of serum with its high levels of ribonucleases may be crucial in the study of this material.

[4] Hansbrough, J. F. Unpublished observation.

The presence in eukaryotic cells of metabolic regulators, governors, etc. has been postulated largely by analogy with microbial systems (27). There is little direct evidence about the chemical nature of these presumed regulators. Our data on the RNase sensitivity of AIM raises the possibility in this lymphoid system of regulation by a species of RNA.

I wish to thank Dr. Albert H. Coons for his interest in this work and Mr. Leo Levine (Massachusetts State Department of Public Health, Boston, Mass.) for supplies of diphtheria and tetanus toxoids. But foremost I acknowledge my indebtedness to Miss Martha Blanchard for her invaluable technical assistance.

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