MATURATION OF THE IMMUNE RESPONSE IN VITRO

Focal Fluctuation and Changes in Affinity of Anti-β-D-Galactosidase Activating Antibody*

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The phenomenon of maturation of the immune response characterized by a progressive increase of the affinity of the antibodies synthesized (1–4) may turn out to be a critical episode for the assessment of the genetic mechanism of differentiation of immunocytes. The mechanism of maturation is not known and the most widely held hypothesis to explain it is that preexisting clones of antigen-sensitive cells are selected progressively according to their capacity to bind antigen (3–5). This implies that all or the majority of cells of a given specificity will respond when the antigen is in excess while only the "best" cells will be stimulated if the antigen becomes limiting. According to such a process, resembling mutant selection in bacteria, one would predict that the disfavored immunocytes would be absent or drastically diluted by the end of maturation. This, however, seems not to be the case because a similar pattern of maturation, with the same starting and end points, is observed during the secondary immune response provided the concentration of immunocytes is reduced (6). Unfortunately there is at present little experimental information to provide an alternative hypothesis. It is not known to what extent maturation can be accomplished in vitro, at the level of a restricted number of clones, in the absence of a continuous cell recruitment from central sources (thymus, bone marrow) and from the circulating pool; and the precise role of antigen in maturation has not been established.

A direct way to the investigation of the mechanism underlying maturation would consist of physical separation of functional clones and then testing of their capacity to undergo maturation under calibrated antigen pressure. To approach this problem we have adopted an in vitro system allowing time-course studies of the immune response, avoiding the regulatory mechanism acting in vivo and allowing direct control of the residual antigen and of the

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antibody synthesized during several weeks.[1] The antigen chosen, β-D-galactosidase (*Escherichia coli*), allows the titration of both precipitating and activating antibodies. The latter are detected by their capacity to increase the enzymatic activity of a defective enzyme, point mutant β-D-galactosidase activable by antibodies (AMEF)[2] (7, 8). Activation of AMEF depends upon antibody binding to a single antigenic determinant (7, 8) which appears to be in a hapten-carrier relationship with the other sites of the molecule.[3] This reaction permits a direct assessment of the affinity of the activating antibody (9). In the current paper we examine: (*a*) the degree of fluctuation of the response reached by decreasing the number of cells per culture, and (*b*) the capacity of maturation in vitro and the effect of antigen dose on the affinity of antibodies synthesized in microcultures.

Materials and Methods

Fragment Culture Technique.—Basically a method of culturing lymph node fragments described by Michaelides and Coons (10) was applied with some modifications. Rabbit lymph nodes were removed aseptically and cut under sterile conditions into fragments containing between 35 and 70 × 10^4 cells. Only one fragment was placed into each culture tube in most experiments, but difragment cultures were also set up as will be indicated when pertinent. In all cases multifragment cultures were prepared as "positive" controls and for comparisons of antibody titers, frequency of response, etc., with the oligofragment cultures. Challenge was performed at 37°C, and later on the fragments were washed six times and placed into the culture tubes as described previously (9). Washings and incubations were performed in Parker 199 medium (Statens Bakteriologiska Laboratoriet, Stockholm, Sweden) containing antibiotics (100 μg/ml streptomycin and 100 units/ml penicillin) (PA). Complete medium (PA + 20% normal rabbit serum) was added, and the cultures were incubated at 37°C. Samples were collected every 3–6 days by pouring the used medium into small sterile tubes. Immediately thereafter, cultures were refed with new medium adjusted to 37°C. Samples were stored at −20°C.

Antigens.—Preparation of wild-type β-D-galactosidase (*E. coli*) (henceforth referred to as β-D-Gal) and the mutant enzyme (AMEF) was performed as described (11).

Rabbits and Immunization.—White rabbits weighing 2–4 kg were purchased from commercial suppliers. Two main different ways of immunization were used: 3 mg of β-D-Gal in complete Freund's adjuvant (CFA) or 1 mg of enzyme in incomplete Freund's adjuvant (IFA) per rabbit. Only one injection of the antigen emulsion was done in one hind footpad, so the corresponding popliteal lymph node was considered the "draining" node and the contralateral popliteal was taken as the "nonregional" lymph node. In some instances axillary nodes were used also as nonregional organs.

Quantitative Precipitation and Coprecipitation Assays.—150 enzyme units (EU) of β-D-Gal were added to each of a series of test tubes containing the same volume of progressive dilutions of the sample to be tested. The mixture was incubated 30 min at 37°C and then 30 min at 0°C. A centrifugation followed at 15,000 rpm for 5 min in a Beckman/Spinco Centrifuge Type 152

[1] Macario, A. J. L., E. Conway de Macario, and F. Celada. Unpublished manuscript.

[2] *Abbreviations used in this paper:* AcT, activating antibody titer; AMEF, point mutant β-D-galactosidase activable by antibodies; CFA, complete Freund's adjuvant; CoPrT, coprecipitating titer; EU, enzyme units; β-D-Gal, β-D-galactosidase of *E. coli*; IFA, incomplete Freund's adjuvant; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; PA, Parker medium with antibiotics; PrT, precipitating antibody titer.

[3] Duplan, J. F., and F. Celada. Unpublished observations.

"Microfuge" (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif). 50 μl of the supernatant were assayed for enzymatic activity (11). The per cent EU precipitated was calculated with the formula:

$$\text{EU/ml added} - (\text{EU/ml supernatant})/(\text{EU/ml} \times 100).$$

The precipitating antibody titer (PrT), expressed as EU precipitated by 1 μl sample, was calculated from the sample dilution giving a precipitation closest to 50%.

The coprecipitation test was performed in the same way up to the second incubation (at 0°C). At this point before the centrifugation, 0.05 ml of goat anti-γ-globulin (Hyland Laboratories, Los Angeles, Calif.) was added to the mixture and incubated 1 hr at 0°C. The percentage of β-D-Gal remaining in the supernatant after centrifugation was calculated as described above. The 50% coprecipitation point was interpolated graphically by plotting on a probability scale, per cent EU precipitated vs. log antibody concentration, whereby a straight line was obtained. The coprecipitation titer (CoPrT) was then calculated and expressed as EU coprecipitated by 1 μl of sample.

Titration of Activating Antibodies.—This enzymatic microassay is based on the enzyme activation test described previously (11). 50 μl of the sample dilution were admixed to 0.02 ml of a fresh preparation of AMEF and incubated at 37°C for 2 hr avoiding evaporation. For the enzymatic assay 2 ml of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) equilibrated to 37°C were added and the mixture agitated. The test tubes were kept at 37°C until a visible yellow color developed, and the reaction was stopped by adding 0.5 ml of 1.2 M CO_3Na_2 and immersing the test tubes in crushed ice. The OD 420nm of the mixture was then measured and EU per milliliter calculated (11). The activating capacity was calculated applying the formula:

$$(\text{EU/ml})_{\text{Exp}} - (\text{EU/ml})_{\text{Background}} = \text{A.EU/ml},$$

where Exp is the enzymatic activity of the mixture (sample dilution + AMEF), and Background is the hydrolytic capacity of the sample + native enzymatic activity of AMEF.

Activating antibody titers (AcT) were determined by making complete activation curves using series of twofold dilutions of the samples. Since the ascending portion of the curve has a slope of ~1 the titer was calculated from any point in this region by the formula:

$$\text{A.EU/ml} \quad \text{dilution factor}.$$

The AcT represents the number of EU over the background produced by 1 ml of undiluted sample acting on 1 ml of AMEF solution.

Micromethod for Determination of Antibody Affinity.—This was performed essentially in the same way as previously described (9). A given concentration of sample (the one able to activate the AMEF preparation up to only 1% of its total activability) was confronted with varying concentrations of antigen (AMEF) and the binding capacity was determined in analogy with determinations of enzyme constants. The activation tests were performed as described above, the main variant being that a given sample (in the dilution chosen) was admixed to a series of progressive dilutions of AMEF. The binding constant was expressed as Km in $(\text{moles per liter})^{-1}$ (the AMEF concentration at which half of the antibody-combining sites are bound) and calculated from a double reciprocal plot 1/(A.EU/ml) vs. 1/AMEF. Linearity of this function indicates homogeneity of the antibodies (in terms of affinity). For nonlinear functions the heterogeneity index was determined by raising the values of [AMEF] to the fractional exponent α (from 0.1 to 0.9). The value of α yielding a modified curve $1/(\text{A.EU/ml})$ vs. $1/[\text{AMEF}]^{\alpha}$ closest to the straight line was taken as the heterogeneity index (12) (see Fig. 1).

Experimental Design.—The experiments reported in this paper were performed according to the following schedule:

(*a*) The prospective donor rabbits were primed.

(*b*) When the primary response had waned (usually after 6 months) the draining and non-regional lymph nodes were removed.

(*c*) Each lymph node was divided into several portions which were identified and then subdivided into microfragments of the desired size.

(*d*) Individual microfragments were challenged in vitro by exposing them to the chosen antigen dose during the desired time interval. This operation was carried out before placing the explants in the culture tubes.

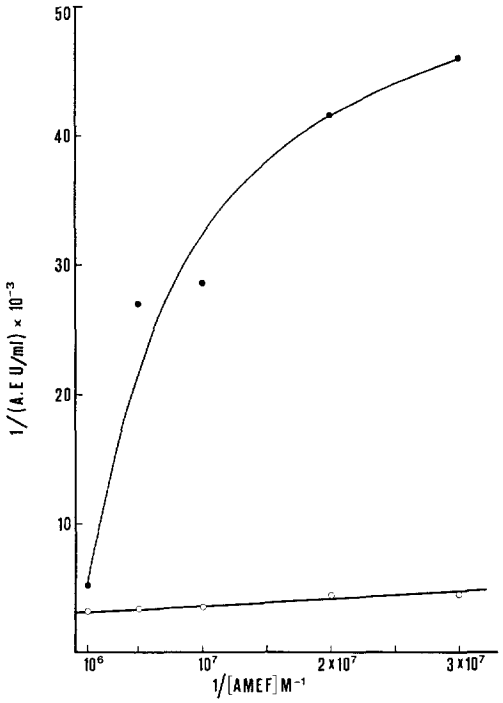

FIG. 1. Example of determination of *Km* of activating antibody using double reciprocal plot. The patterns corresponding to two different samples of the same culture are shown. ●——●, antibodies collected at day 11 of cultivation; the heterogeneity index calculated according to Sips is 0.6 and the binding constant is 11^4 M^{-1}. ○——○, antibodies collected at day 41 of cultivation; the heterogeneity index is 1 and the binding constant is 5.7×10^7 M^{-1}.

(*e*) Microfragments were cultivated for periods ranging between 1 and 3 months. Used medium was changed at regular intervals (3–6 days) and kept for antibody determinations.

(*f*) Precipitating, coprecipitating, and activating antibody titers were determined; the affinity and degree of heterogeneity of the latter antibodies were measured in successive samples of all cultures; and profiles of the immune response were constructed.

RESULTS

I. Discontinuity of the Response of Series of Microfragments from a Single Lymph Node.—In a series of experiments it was demonstrated that the fre-

quency of response of microcultures is dependent upon the number of fragments (i.e., the size of the inoculum) per tube. Multifragment cultures (8–10 fragments, 5×10^5 or more cells) were 100% positive. By decreasing the number of fragments per culture the frequency of responder cultures decreased and this trend was more pronounced when nonregional lymph nodes were used. Figs. 2 and 3 illustrate the frequency distribution, in terms of activating antibody titer, at the peak of the response of microcultures containing either one (*b*) or two (*a*) microfragments from the draining node (Fig. 2) and from

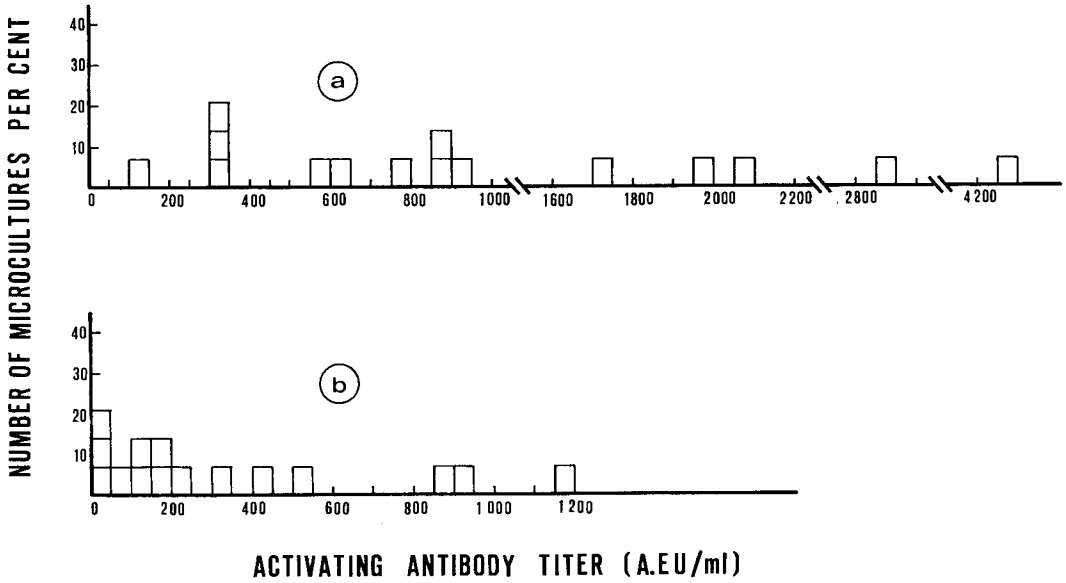

FIG. 2. Frequency distribution of microcultures in terms of activating antibody titer at the peak of the secondary response. Each rectangle represents one culture that contained either one (*b*) or two (*a*) microfragments from a draining lymph node.

the nonregional lymph node (Fig. 3) of a single donor. The experiment shown had a relatively high frequency of responder fragments and this emphasizes that discontinuity of response can always be reached by reducing the number of cells per culture. Difragment cultures of the draining lymph node were all positive and only 30% of them had AcT over 1000 A.EU/ml, that is, the minimal level reached by all the control multifragment cultures. The majority of the cultures had intermediate AcT, between 300 and 1000 A.EU/ml. In the case of monofragment cultures 80% were positive, only 6% had AcT over 1000 A.EU/ml, and 40% clustered in the region of lowest AcT. Comparison of Figs. 2 *a* and *b* shows that a drastic reduction in the frequency of response and antibody levels was accomplished when the number of cells per tube was reduced to one-half.

The data of cultures of nonregional fragments (Fig. 3) showed that in the case of difragment cultures nearly 35% of them were negative, only 6% had AcT over 1000 A.EU/ml, and more than 50% clustered between 50 and 300 A.EU/ml. Monofragment cultures exhibited the following features: 50% of them were negative, half of the responder cultures accumulated in the lowest AcT region, and none had AcT over 1000 A.EU/ml.

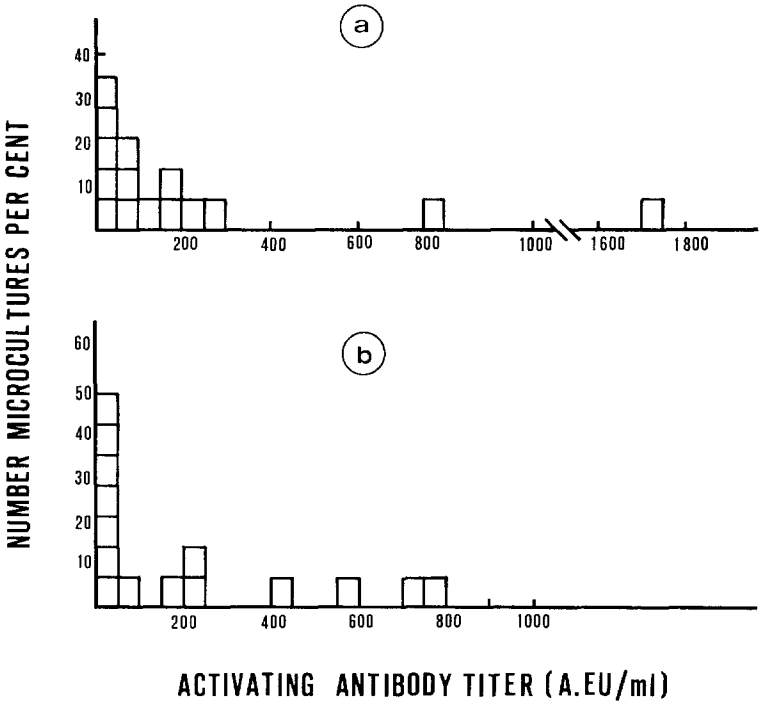

FIG. 3. Frequency distribution of microcultures in terms of activating antibody titer at the peak of the secondary response. Each rectangle represents one culture that contained either one (*b*) or two (*a*) microfragments from one nonregional lymph node belonging to the same donor of the draining node referred to in the legend to Fig. 2.

II. Quantitative Variation of the Secondary Response of Microfragments from Different Rabbits.—The frequency of response and antibody titers of microcultures were also affected by the source of the fragments, e.g., by the preimmunization history of the donor rabbits. The frequency of response was higher in microcultures of fragments belonging to rabbits immunized with 3 mg of β-D-Gal than in microcultures of explants from lymph nodes of rabbits immunized with 1 mg of antigen. One representative experiment is shown in Table I. In some experiments consisting of a small number of microcultures from the draining lymph node presensitized as mentioned above, differences in

frequency were not significant between animals immunized differently, but antibody titers differed strikingly. An example of this situation is given in Fig. 4. The frequency of response was always higher in microcultures of frag-

TABLE I

*Frequency of Positive Cultures of Single Microfragments Belonging Either to Draining or Nonregional Lymph Nodes of Rabbits Primed in Two Different Ways**

Lymph node	Rabbit	
	3‡	1§
Draining	55/60 (92%)	12/20 (60%)
Nonregional	18/30 (60%)	6/35 (17%)

$P < 0.01$ for 3 (draining) vs. 1 (draining). $P < 0.001$ for 3 (nonregional) vs. 1 (nonregional); 3 (draining) vs. 3 (nonregional); 1 (draining) vs. 1 (nonregional).

* Results at the peak of the activating antibody response (cultures negative at this moment were nonresponders). Frequency is expressed as cultures positive/cultures investigated. Control cultures not challenged were negative.

‡ Rabbit 3: primed with 3 mg of β-D-galactosidase (*E. coli*) in complete Freund's adjuvant.

§ Rabbit 1: primed with 1 mg of the same antigen in incomplete Freund's adjuvant.

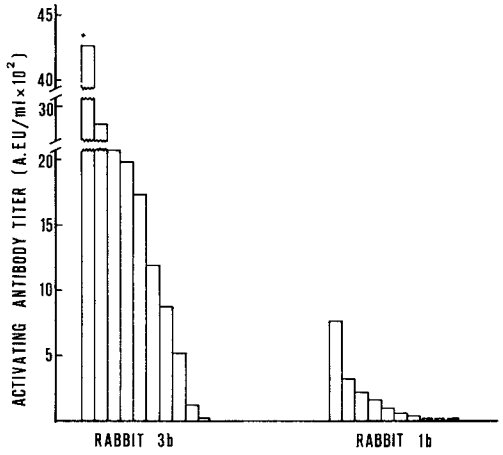

FIG. 4. Influence of the donor rabbit on the peak level of activating antibody synthesized by lymph node microfragments in culture. Each bar represents one fragment belonging to the draining node of a rabbit preimmunized with 3 mg of β-D-Gal in CFA (rabbit *3b*) and of a rabbit preimmunized with 1 mg of the same antigen in IFA (rabbit *1b*). Horizontal lines represent nonresponder cultures.

ments taken from draining lymph nodes, as shown in Table I; differences were sometimes found among portions of the same lymph node (this is shown in Table III).

III. Specificity Fluctuation of Positive Cultures of Microfragments from a

Single Lymph Node.—Multifragment cultures from the same lymph node prepared and challenged under the same conditions gave very similar responses, resembling the pattern of the in vivo response by every parameter tested, as expected for the simultaneous presence of many responder units. Cultures containing fewer cells (one or two microfragments) gave a heterogeneous spectrum of responses; individual cultures differed from each other in several respects: (*a*) activating antibody titer, as shown in part I, (*b*) precipitating antibody titer, from values close to 2000 EU/ml to nondetectable levels, (*c*) coprecipitating titer, from values over 20,000 EU/ml to trace amounts of antibody (see Tables II and III), (*d*) ratio AcT/PrT. Apart from a certain number

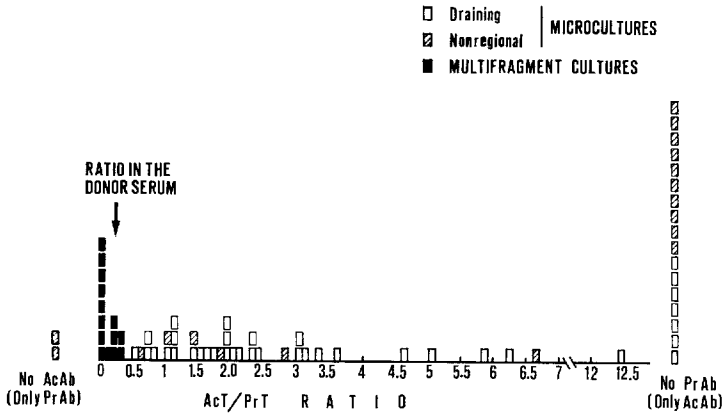

FIG. 5. Distribution of ratios AcT/PrT found in monofragment (open and cross-hatched rectangles) and multifragment cultures (solid rectangles) from the same rabbit. The position of the ratio calculated for the serum of the donor rabbit is marked by an arrow. Microcultures in which only precipitating antibodies (PrAb) were found are shown to the left and those in which antibodies were not detected by the precipitation technique are represented to the right, apart from the scale. The data are from the experiment illustrated in Tables II and III.

of cultures where only activating antibody or precipitating antibodies were produced, the ratio between the two titers varied greatly. Fig. 5 shows the distribution of this ratio in multifragment and in microcultures of one experiment. The values of multifragment cultures had a very narrow distribution and did not differ from the ratio found in the serum of the donor rabbit, whereas the ratios corresponding to the microcultures were dispersed over a 25-fold range. (*e*) Ratio AcT/CoPrT. The trend was similar as for AcT/PrT ratio, but the range of variation was smaller, as expected from the higher coprecipitation titers; however, a certain number of cultures had only activating antibody (e.g. tube 2, Table III).

IV. Influence of Antigen Dosage on In Vitro Maturation of the Immune Response.—In order to study the influence of antigen dosage (as concentration and time of antigen exposure) on maturation of the secondary response of

microfragments a series of culture experiments was performed. Fragments from the draining and one nonregional lymph node of the same rabbit were challenged with different antigen doses. Parallel series of nonchallenged cultures were prepared. Time-course measurements of AcT, PrT, CoPrT, the corresponding ratios, and activating antibody affinity and heterogeneity index according to Sips were carried out. These parameters for one experiment are listed in Tables II and III. The maturation of the immune response in terms of increasing binding constant of activating antibody with time of cultivation is also shown graphically in Fig. 6 where data corresponding to the microcultures of Table II have been plotted. The main features of these data are the following:

(*a*) There was an inverse correlation between the challenging antigen dose and the affinity of early activating antibody.

(*b*) The degree of maturation (both in terms of frequency of maturating cultures and degree of increase in affinity per microculture) rose when the challenging antigen dose was increased from 0.5 μg (where no maturation occurred) to 50 μg. With a challenge of 500 μg difragment cultures showed maturation while monofragment cultures (of which only three out of five responded) did not. Fig. 7 shows the increase in affinity plotted against the antigen challenge dose on a log/log scale. The points represent averages of mono- and difragment cultures. The maturation capacity grows exponentially with the antigen dose, until inhibition of high affinity antibody-forming clones takes place in antigen excess.

(*c*) There was a direct correlation between maturative change in *Km* and increase of heterogeneity index. As a rule only the cultures with low index did mature with time.

(*d*) As far as the relation between maturation capacity and the other parameters listed in Tables II and III is concerned, there was a certain tendency of cultures with higher over-all titers and relatively low AcT/PrT ratio to show great affinity changes.

(*e*) The general picture was similar when results obtained with nonregional lymph nodes were analyzed (see Table III). Compared with draining lymph node pieces the nonregional explants showed a lower frequency of response, which was much lower if only activating antibodies are considered. The affinity of early antibodies was already high with challenge of 0.5 and 5 μg, but Fig. 7 shows that the slope of increase in affinity becomes parallel to that of regional fragments when the challenge dose is raised.

DISCUSSION

The characteristics of the β-D-Gal (*E. coli*) as an antigen and of the immune response in tissue culture directed against this enzyme was described elsewhere.[1] This discussion shall be limited to the results obtained when the size of the lymph node explants cultured individually was reduced to the smallest size (corresponding to a content of $\sim 10^5$ cells) still allowing uniformity of the

TABLE II

*Variability of the Immune Response and Influence of Antigen Dose on Its Maturation in Cultures of Microfragments Belonging to a Draining Lymph Node**

	0.5 μg							5 μg							50 μg							500 μg						
	Tube	Days	*Km* × 10^5	H.I.	AcT	CoPrT	Ratio	Tube	Days	*Km* × 10^5	H.I.	AcT	CoPrT	Ratio	Tube	Days	*Km* × 10^5	H.I.	AcT	CoPrT	Ratio	Tube	Days	*Km* × 10^5	H.I.	AcT	CoPrT	Ratio
1F	1	11	4.1	0.8	1.17	8.08	0.13	11	16	0.72	0.7	1.26	5.22	0.19	21	11	0.23	0.6	0.40	1.44	0.22	31	11	0.08	0.6	0.13	1.55	0.08
		31	6	0.8					31	52	0.9					22	30	0.9					22	0.08	0.6			
	2	19	4	0.8	0.10	±	1	12	16	0.8	0.7	0.63	4.35	0.13	22	11	0.19	0.6	0.37	1.51	0.22	32	11	0.56	0.7	0.6	0.42	0.12
		26	4	0.8					31	14	0.9					22	20	0.9					19	0.56	0.7			
	3	11	2.8	0.8	0.07	0.25	0.22	13	16	23.8	0.7	0.42	2.03	0.17	23	16	0.7	0.7	0.67	1.29	0.34	33	11	0.12	0.6	0.56	1.97	0.22
		19	3	0.8					22	63.0	1					26	1	0.7					26	0.18	0.6			
	4	11	13	0.8	0.68	9.30	0.07	14	16	0.28	0.7	0.31	2.23	0.12	24	16	1.78	0.7	0.86	2.51	0.26	34				—	0.78	
		26	11	0.8					19	0.28	0.8					22	6	0.8										
	5	11	6	0.8	0.48	2.90	0.14	15	16	1.70	0.7	0.17	2.40	0.07	25	16	0.23	0.6	1	3.01	0.25	35				—	0.72	
		22	5	0.8					26	18.00	0.9					22	1.12	0.7										
2F	6				—	0.62		16	16	0.16	0.7	0.92	4.11	0.18	26	16	0.2	0.7	0.84	1.44	0.37	36	11	0.11	0.6	0.65	2.60	0.20
									31	2	0.8					31	4	0.8					41	573	1			
	7	11	4.8	0.8	0.73	3.40	0.18	17	16	1.6	0.8	0.98	5.53	0.15	27	11	0.8	0.7	0.66	4.09	0.14	37				—	0.75	
		26	4.8	0.8					22	25	0.9					22	32	0.9										
	8				0.14	0.46	0.23	18	16	3.4	0.8	1.02	5.87	0.15	28	16	5	0.8	1.51	4.91	0.24	38	11	0.16	0.6	1.67	9.35	0.15
									26	7	0.8					26	7.3	0.8					31	4	0.8				
	9	11	40	0.9	0.84	4.02	0.17	19	16	0.22	0.7	2.33	6.32	0.27	29	16	1	0.7	1.07	3.40	0.24	39	11	0.3	0.6	0.13	0.41	0.24
		31	40	0.9					41	14.4	0.9					31	50	0.9					41	6	0.8			
	10	11	11.9	0.8	0.84	4.62	0.15	20	16	0.28	0.7	0.66	4.02	0.14	30	16	1.2	0.7	2.01	7.52	0.21	40	11	0.24	0.6	0.87	1.51	0.37
		31	22.0	0.9					22	2.3	0.8					41	44	0.9					31	6	0.8				

* The donor rabbit was immunized with 3 mg of β-D-Gal in CFA, 9 months before starting the cultures. Challenge at time 0 was performed with 0.5, 5, 50, and 500 μg of β-D-Gal, as indicated. Multifragment cultures were 100% positive for activating and precipitating antibodies (not shown). Cultures not challenged were negative for both types of antibodies (not shown). *Tube:* microculture number. *Days:* time of cultivation. *Km:* in (moles per liter)$^{-1}$ *H.I.:* heterogeneity index. *AcT:* activating antibody titer at the peak of the response. *CoPrT:* coprecipitating titer at the peak of the response. *Ratio:* AcT/CoPrT. *1F:* One lymph node fragment per microculture. *2F:* two fragments per microculture.

TABLE III

*Variability of the Immune Response and Influence of Antigen Dose on Its Maturation in Cultures of Microfragments Belonging to a Nonregional Lymph Node**

	0.5 μg							5 μg							50 μg						
	Tube	Days	*Km* × 10^5	H.I.	AcT	CoPrT	Ratio	Tube	Days	*Km* × 10^5	H.I.	AcT	CoPrT	Ratio	Tube	Days	*Km* × 10^5	H.I.	AcT	CoPrT	Ratio
1F	1				—	0.18		11				—	—		21	7	0.24	0.6	0.41	0.81	0.32
																19	600	1			
	2	11	90	1	0.09	—	1	12	7	40	0.9	0.22	1.06	0.17	22	11	6	0.8	0.24	0.38	0.39
		15	93	1					19	60	0.9					19	600	1			
	3				—	—		13				—	2.45		23	11	7	0.8	0.20	1.75	0.10
																19	100	1			
	4	11	130	1	0.72	2.33	0.24	14				—	0.36		24				—	0.72	
		26	206	1																	
	5				—	—		15	11	60	0.8	0.79	7.83	0.09	25	11	0.8	0.7	0.60	1.96	0.28
									31	70	0.9					22	6	0.8			
2F	6	11	88	1	0.11	2.50	0.42	16				—	0.41		26	11	2.48	0.7	0.85	7.76	0.10
		26	88	1												31	1062	1			
	7	7	80	1	1.72	20.40	0.08	17	11	80	0.9	0.20	1	0.17	27						
		31	180	1					22	280	1								—	0.67	
	8	11	15	0.9	0.17	0.25	0.40	18				—	0.36		28				—	1.56	
		19	28	0.9																	
	9	11	350	1	0.21	1.90	0.10	19				—	0.31		29	15	11	0.8	0.05	0.97	0.05
		31	440	1												19	430	1			
	10	11	20	1	0.06	0.27	0.18	20				—	1.01		30	15	300	1	0.26	2.63	0.09
		22	10.3	1												19	300	1			

* Cultures were prepared with microfragments from the nonregional lymph node of the rabbit mentioned in the footnote to Table II. Challenge at time 0 was performed with 0.5, 5, and 50 μg of β-D-Gal, as indicated. Multifragment cultures were 100% positive for activating and precipitating antibodies (not shown). Cultures not challenged were negative for both types of antibodies (not shown). Other references can be read in the footnote to Table II.

number of cells per fragment. The following distinctive facts were observed in series of "identical" cultures (prepared with fragments of the same size from a single lymph node, challenged, and kept in culture under the same conditions): (*a*) fluctuation of positivity for secondary anti-β-D-Gal response, (*b*) wide variation in antibody titers, (*c*) oscillation of antibody specificity composition of the culture fluids, (*d*) differences in affinity of early activating antibodies and changes in affinity with time.

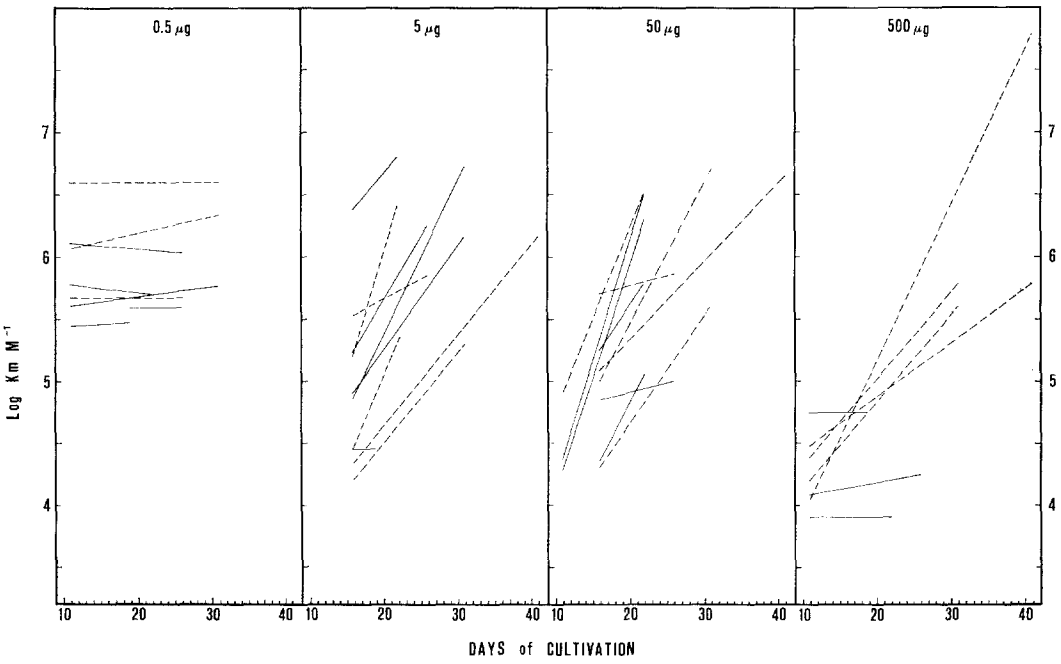

FIG. 6. Maturation of the activating antibody response by microcultures challenged at time 0 with 0.5, 5, 50, and 500 μg of β-D-Gal. Each line represents one microculture (the broken lines are for difragment cultures) and joins the *Km* values of activating antibody present in the earliest and latest samples tested.

The frequency of nonresponder cultures is greatest if activating antibody production is considered. This is consistent with the suggestion[1] that antibodies binding to any determinant of the antigen can be detected by the coprecipitation test, while AMEF activation detects only antibodies directed towards a determinant expressed once on each enzyme molecule. The simple precipitation probably recognizes the same spectrum of antibodies as the coprecipitation, but its sensitivity, and therefore its usefulness in determining true nonresponders, is limited by the characteristic "antigen excess" zone where no precipitation occurs. The considerable fraction of 10^5-cell cultures negative for

activating antibody, together with the wide quantitative oscillation of the positive ones, indicates that a size of fragments has been reached where the number of functioning clones per fragment is very small (0, 1, 2, 3, . . .). There is no evidence, however, that antigen-sensitive memory cells are disseminated at random in the lymph node tissue and therefore we cannot assume that they are distributed in the cultures according to Poisson. This prevents us from calculating the expected frequency of cultures containing one single clone or focus. Another signal that focal level has been reached is the independent segregation of the capacities to respond to different specificities. This results in fluctuation of the ratio of activating antibody to precipitating antibodies, which is much wider in the microcultures (~100,000 cells) than in multifragment cultures (~1 million cells). The range of fluctuation would certainly have

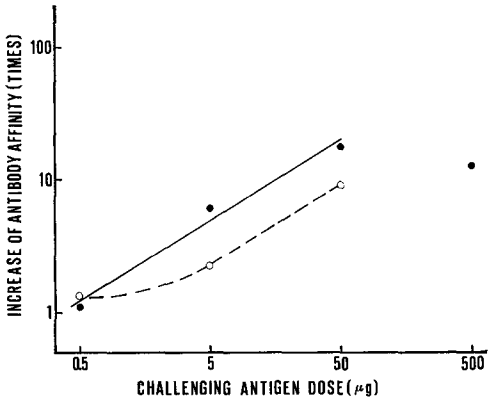

FIG. 7. Exponential increase of activating antibody affinity of the in vitro secondary response relative to the challenging antigen dose at the beginnig of the cultures. The data shown are listed in Tables II and III.

been still wider if two completely independent responses had been compared; instead it is probable that activating antibodies may contribute to the precipitation reaction, even if pure activating antibodies do not precipitate.

While other authors have measured the affinity or avidity of antibodies released in vitro (2, 13, 14) the maturation of the immune response in tissue culture has not been described except for our previous communication (9). According to the present results obtained with optimal challenge, the maturation in vitro is similar to what happens during the primary response in the intact animal (1, 2, 14–16), or in the secondary adoptive response with a small number of transferred cells (6), in terms of range and kinetics of the excursion of affinity measurements in "early" and "late" antibodies. If instead a limiting or excessive dose of antigen is administered the early antibodies are already remarkably homogeneous, with high or low affinity respectively, and no maturation occurs during the weeks of observation. It should be noted, however,

that compared with the in vivo response, our culture conditions avoid the feedback and competitive inhibition by performed circulating antibodies and limit the competition among clones with different affinity since the size of the fragments is small and they are challenged and cultured individually. In these respects microcultures are similar to adoptive transfers of small cell numbers and the discrepancy between our results and those of others (3, 13) showing that early antibodies elicited by secondary challenge are already of high affinity may be explained on the same grounds (6). Moreover, by changing the whole tissue culture medium at short intervals the intermixing of antibodies produced at different stages of the response is minimized.

It is possible to interpret the present results according to the clonal selection theory along the lines suggested for the maturation in vivo (4). 10^5-cell fragments would contain several predetermined cell lines making up a wide spectrum of different antibody affinities. Either a few (at limit only one) or all of them would be activated, depending on the amount of available antigen, which is necessary for both initiation and continuation of the antibody production. The decrease of the antigen would allow only the clones with highest affinity to continue to function. Excess of antigen would cause paralysis of high affinity antigen-sensitive cells, so that only homogeneous low affinity antibodies could be produced upon secondary challenge under the experimental conditions described in this paper in spite of the fact that the donor rabbit had reached a high affinity and homogeneity of its serum antibodies at the end of the primary response. Therefore a corollary of the above-mentioned interpretation is that at variance with the high rate antibody-forming cells no affinity selection is operating at the level of the memory cells (6). On the other hand the required presence of a battery of clones directed towards a single determinant in all microfragments is difficult to reconcile with the evidence discussed above, suggesting that the level of small numbers has been reached. An alternative possibility is that some sort of antigen-controlled evolution of affinity may occur within one cell line endowed with the capacity to recognize a given specificity, also after it is cut off from contacts with the circulating pool of lymphocytes and from the central organs (thymus, bone marrow). Such an explanation would be favored by the data in Fig. 7 where the exponential increase of maturation potential with antigen challenge, apparently independent of the number of memory cells initially present (certainly more numerous in draining than in nonregional fragments), suggests a continuous generation of "new affinities." An independent control of the purity of apparently monoclonal cultures, e.g. by allotype markers, is needed before this dilemma can be resolved. Experiments in this direction are presently being performed in our laboratory.

SUMMARY

We have cultivated lymph node microfragments from β-D-galactosidase (*Escherichia coli*) primed rabbits and have measured their secondary response

directed towards the whole molecule (precipitating antibodies) and to a single determinant (activating antibodies) of the antigen. By decreasing the size of the fragments to 10^5 cells, we began to observe heterogeneity among identical cultures in terms of positivity of response, antibody specificity, and titers. The affinity of "early" activating antibodies was inversely proportional to the dose of challenge. While no maturation was seen in low and excessive challenge, in all cultures receiving intermediate doses the association constant was raised several orders of magnitude within periods of 20 days. The relevance of these data to the mechanism of affinity selection of antigen-sensitive cells is discussed.

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