STUDIES ON THE REGULATION OF AVIDITY AT THE LEVEL OF THE SINGLE ANTIBODY-FORMING CELL*

The Effect of Antigen Dose and Time after Immunization

By BIRGER ANDERSSON, M.D.

(*From the Department of Tumor Biology, Karolinska Institutet, Stockholm, Sweden*)

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The cellular events operating in the regulation of the affinity[1] of antibodies produced during immunization and the response to different immunizing doses of antigen require further investigations. So far most of the experiments in this field have been performed at the level of the intact animal and the impact of antigen dose, time after immunization, feedback inhibition by passive antibody, and induction of partial tolerance have been studied by determinations of the affinity at the serum level.

Essentially the findings in this field are: the antigen concentration seems to exert a selective pressure at the cellular level in the immune response, so that cells producing antibodies with a relatively low affinity for the antigen need a higher concentration of antigen to become stimulated into antibody formation than cells producing high affinity antibodies. Findings that have been interpreted as consequences of such selective processes are: (*a*) the gradual increase in affinity with time after immunization (1); (*b*) the high doses of antigen initiating the production of antibodies of lower affinity than that of antibodies produced as a response to a low dose (1, 2); (*c*) the relative resistance of high affinity antibody production to feedback inhibition by passive antibody (2); (*d*) the fact that high affinity antibody is more efficient in feedback inhibition than low affinity antibody (3); (*e*) the fact that partially tolerant animals produce antibody with relatively low affinity, i.e., the high affinity cells are more easily paralyzed than the low affinity cells (4, 5).

All the facts and conclusions cited above postulate a similar binding constant (or an identity) for the cell-associated antigen-specific receptor and the product of the cell released as a result of the combination of the antigen and the receptor. So far the only direct evidence for such a mechanism is the finding that there is a correlation between the affinity of the antibody produced by immune-cell donor animals and the concentration of antigen needed to trigger the immune cells into mitosis in vitro (6).

The hemolytic plaque assay (7, 8) constitutes an excellent tool to analyze the cellular events in the immune response, since it is possible at a given time to quantitate the number of cells producing antibody against a given antigen specificity. Modifications

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[1] Affinity is the average binding constant in an antibody-hapten system. Avidity is the the tendency of an antibody sample to form stable complexes with a macromolecular antigen.

have been invented that make it possible to study cells forming antibody against a variety of proteins (9–11), polysaccharides (12, 13), synthetic polymers (14), and haptens (15, 16). The use of specific developing sera for plaque-forming cells (PFC:s) allows the separate detection of cells producing antibodies belonging to different immunoglobulin classes (17, 18) or allotypes (19, 20).

In the present article the hemolytic plaque assay has been adapted to the study of avidity[1] at the level of the single antibody-forming cell. The method is based upon the principle that hemolytic plaques against sensitized erythrocytes are inhibited by free antigen. The inhibition characteristics of the plaques reflect the avidity of the antibodies released by the single hemolytic plaque-forming cells (PFC:s).[2] The impact of the immunizing dose and of the time after immunization has been studied with regard to the avidity both in the serum and at the cellular level.

Material and Methods

Animals.—Adult inbred mice of (C3H × C57BL)F_1 genotype were used. In each experiment animals of the same age and sex were used.

Immunization.—The mice were immunized by an injection of 0.05 ml of an emulsion of Freund's complete adjuvant containing 50 or 5000 μg of bovine serum albumin (BSA) (Mann Research Labs, Inc., N.Y., Fraction V), ovalbumin (OA) (Worthington Biochemical Corp., Freehold, N.J., 3× crystallized), or human serum albumin (HSA) (Kabi, AB, Stockholm, Sweden) into each rear footpad.

Serologic Tests.—BSA was radioiodinated by the chloramine-T method (21). Radioiodinated HSA was purchased from Radiochemical Centre, Amersham, England. The antigen-binding capacity (ABC) of antisera was determined at 50% binding of antigen by the Farr ammonium sulfate method (22) using as standard concentrations 1 and 100 μg of antigen per ml during the test. The factor of avidity was calculated according to Celada et al. (5) using the formula:

$$\log \mu l \text{ As} = m + s \log \mu g \text{ Ag},$$

where s is the slope of the line obtained when microliters of antiserum needed to bind 50% of antigen are plotted on a log/log scale against μg antigen in the reaction mixture. s is the factor of avidity and varies between 0 and 1. A maximal avidity is indicated by values near 1. m is the intercept with the line $\log \mu g \text{ Ag} = 0$.

Bleeding of Animals and Preparation of Cell Suspensions.—The mice were bled from the retro-orbital sinus and thereafter killed by cervical dislocation. The regional popliteal lymph nodes were harvested in Eagle's medium in Earle (EME) and single cell suspensions were prepared by cutting and squeezing the nodes followed by decantation and washing by centrifugation in EME. The suspensions were kept in ice bath until tested.

Assay for Single Antibody-Forming Cells.—Antibody production against BSA, HSA, or OA at the single cell level was studied by the hemolytic plaque assay (7, 8). Target cells were sheep erythrocytes coated with the respective albumin by the use of bis diazotized benzidine (10). In order to obtain formation of clear plaques, a concentration of 5 mg of albumin per ml

[2] Abbreviations used in this paper: ABC, antigen-binding capacity; BSA, bovine serum albumin; EME, Eagle's medium in Earle; HSA, human serum albumin; OA, ovalbumin; PBS, phosphate buffered saline; PFC:s, single hemolytic plaque-forming cell.

was necessary during the coupling procedure. A rabbit–anti-mouse immunoglobulin serum at a dilution of 1:50 was used to develop the plaques (10). In this system only "developed" plaques produced by 7S antibody-releasing cells were obtained and the serum antibodies are found only in the IgG region as shown by gel filtration.[3]

RESULTS

Inhibition of Plaque Formation by Free Antigen—Inhibition of plaque formation can be expected to occur if fluid antigen is present in the agar in which the hemolytic plaque formation takes place. The free antigen would compete for plaque-producing antibodies with the antigen attached to the target erythocytes. This hypothesis was tested by adding 0.1 ml of phosphate buffered saline (PBS) containing dissolved antigen to the liquid agar which was used as the top layer immediately before plating. The per cent inhibition was calculated from control plates to which 0.1 ml of PBS without antigen had been added. Calculations were made from mean values of two to four plates for each amount of antigen per plate.

TABLE I

Specificity of the Inhibition of Hemolytic Plaque Formation by Free Antigen

Immunizing antigen	Inhibitor μg per plate	No. of plagues per aliquot of cells	
		BSA-coated SRBC	OA-coated SRBC
BSA	0	416; 492	3; 7
	BSA, 1000	0; 0	2; 2
	OA, 1000	776; 548	1; 1
OA	0	0; 1	824; 796
	BSA, 1000	0; 0	624; 552
	OA, 1000	0; 0	1; 1

Table I shows that specific inhibition can be obtained in this way. A concentration of free BSA that completely inhibited the plaque formation against BSA-coated erythrocytes did not decrease the plaque formation against OA, and vice versa. Fig. 1 shows a typical inhibition curve obtained by adding increasing amounts of free BSA to the plates in the anti-BSA system. The experimental variation is indicated by the standard errors of the means. In order to express in a simple way the differences in inhibition characteristics between different plaque-forming cell (PFC) populations, we calculated the concentration of antigen in the agar needed to obtain 50% inhibition.

Detection of Avidity at the Cellular Level.—The specific inhibition curves obtained by adding increasing amounts of dissolved antigen to the agar in the hemolytic plaque system (Fig.1) is presumably caused by the formation of

[3] Andersson, B., unpublished findings.

relatively stable complexes between the free antigen and the antibody released by the PFC:s. It is logical to assume that the binding constant for the reversible antigen-antibody reaction would influence the antigen concentration necessary to neutralize the antibodies so that the hemolytic plaque formation is inhibited, i.e., the antibodies are prevented from reaching the antigen attached to the erythrocytes. This assumption was tested by comparing the inhibition curves obtained with PFC:s from donor animals showing different avidity of their serum antibodies. It can be seen in Fig. 2 that a marked difference in inhibition characteristics was obtained. PFC:s from animals with low avidity serum antibodies required a higher antigen concentration in the agar to become inhibited than the high avidity PFC:s. In order to test whether these differ-

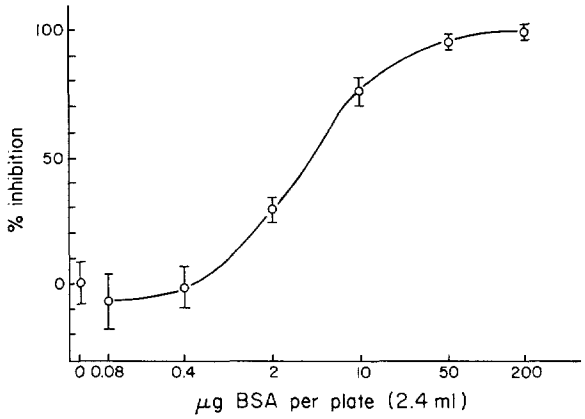

FIG. 1. Specific inhibition of hemolytic plaque formation against BSA-coated erythrocytes. Each point is the mean of four plates and vertical bars indicate SE.

ences really were reflecting the avidity, antibodies released by the PFC:s were first allowed to attach to the target erythrocytes and thereafter elution by free antigen was performed. The outcome of such an experiment is shown in Fig. 3. It is obvious that the plaques that are more easily inhibited by antigen incorporated into the agar are also more resistant to elution by free antigen added to the plates after cellular release of antibody. These findings are in perfect agreement with the concept that the described inhibition method detects differences in the avidity for the antigen at the cellular production level.

The Effect of Time after Immunization upon the Avidity of Antibodies Released by PFC:s—A gradual increase in avidity or affinity with time after immunization has been reported in a variety of different systems (1, 22–25). It was therefore investigated whether such an impact of time after immunization could be detected also in the present, single cell system. In Tables II–IV it is shown that the avidity of anti-BSA and anti-HSA antibodies released by PFC:s increases with time after immunization. Serum antibody determinations were

also made and the avidity in the serum increased in parallel with the avidity at the PFC level. It should be noted here that only PFC:s from the regional lymph nodes were tested, whereas serum antibody also is contributed to by production from other loci, such as spleen and other lymph nodes.

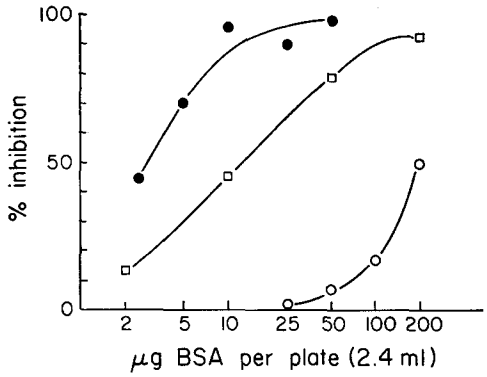

FIG. 2. Specific inhibition of hemolytic plaque formation against BSA-coated erythrocytes. A comparison between PFC populations from animals with different avidity of their serum antibodies. O——O serum factor of avidity 0.17, □——□ serum factor of avidity 0.63, ●——● serum factor of avidity 0.97. Each PFC sample is a pool from the regional lymph nodes of four animals. The serum avidity was determined on the pooled sera from these animals. Each point is the mean value of duplicate or triplicate plates.

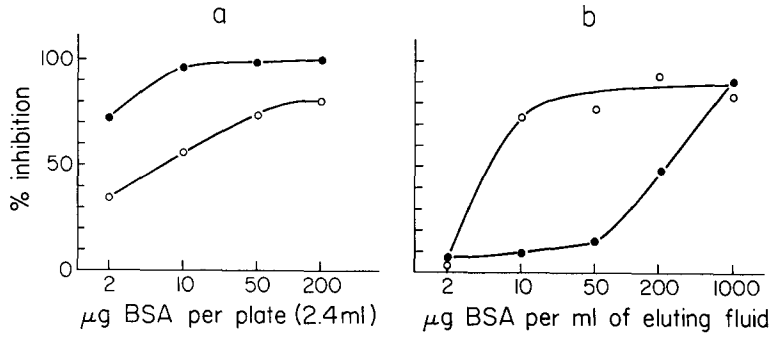

FIG. 3. Specific inhibition of hemolytic plaque formation against BSA-coated erythrocytes. A comparison between the effect of (*a*) having the antigen incorporated in the agar during the period of antibody release from the PFC:s and (*b*) the effect of adding the antigen to the plates after the period of antibody release from the PFC:s. 4 ml of PBS containing 0–1000 μg BSA per ml was added to each plate. ●——● PFC pool from three animals with serum factor of avidity 0.46. O——O PFC pool from three animals with serum avidity 0.39.

The Effect of the Immunizing Dose upon the Avidity of Antibodies Released by PFC:s.—Another parameter which in other systems has been shown to influence the avidity or affinity of antibodies is the immunizing dose (1, 2). In Tables II and III in this article are listed avidity determinations of antibodies

released from PFC:s in animals immunized with 50 and 5000 μg of BSA respectively. It can be seen that at day 7 after immunization, PFC:s from the 50 μg group were inhibited 50% by 6.4 μg BSA/ml agar whereas the PFC:s from the 5000 μg group required 83.3 μg/ml for 50% inhibition. At days 11 and 14 differences can also be seen between the groups but an increase in the avidity in both groups due to the time factor is also observed. At day 30 the

TABLE II

Variations in the Avidity of the Antibody Response of Mice Immunized with 50 μg of BSA into Each Hind Footpad. A Comparison between Serum Antibodies and Antibodies Released by PFC:s

Day	Serum antibodies				Plaque-forming cells	
	ABC‡ at 1 μ/ml	ABC at 100 μ/ml	Factor of avidity		PFC:s in the regional lymph nodes	Agar plates giving 50% inhibition*
						μ antigen/ml
7	11.12	64.95	0.62	0.56	2000	6.40
	5.02	50.0	0.50		1840	
	11.86	84.13	0.57		390	
11	62.4	398.29	0.60	0.56	1160	1.92
	186.0	526.47	0.77		1050	
	25.7	374.71	0.42		265	
	27.8	469.42	0.39		255	
14	97.5	694.3	0.57	0.57	2800	1.50
	121.5	723.65	0.61		1960	
	40.50	410.63	0.50		1820	
	159.9	1050.21	0.59		1780	
30	118.04	126.91	0.98	0.95	1340	0.58
	94.52	129.31	0.93		360	
	29.59	44.40	0.91		360	
	104.23	121.85	0.97		2800	

* The cells from the regional lymph nodes were pooled and tested for inhibition by free antigen.

‡ μg BSA bound per ml of serum.

groups are shown to be practically equalized. The values for the avidity of the serum antibodies are showing similar differences as those recorded for the antibodies released from the PFC:s.

Test for the Presence of Free Antigen in the PFC Suspensions.—The test cell suspensions might contain remaining free antigen in amounts sufficient to interfere with plaque formation and thus create artificial inhibition differences when cells from animals immunized with different doses or immunized at

different times before testing are compared. Table V shows a mixture experiment where we tested whether PFC:s from animals immunized with 5000 μg and showing low avidity both at the serum level and at the cellular production level, interfered with plaque formation when mixed with PFC:s from animals immunized with 50 μg and showing high avidity both in the serum and when the PFC:s were tested. The data gave no indication that the two

TABLE III

Variations in the Avidity of the Antibody Response of Mice Immunized with 5000 μg of BSA into Each Hind Footpad: A Comparison between Serum Antibodies and Antibodies Released by PFC:s

Day	Serum antibodies				Antibody forming cells	
	ABC‡ at 1 μ/ml	ABC at 100 μ/ml	Factor of avidity		PFC:s in the regional lymph nodes	Agar plates giving 50% inhibition*
						μ antigen/ml
7	0.62	20.52	0.24	0.17	195	83.30
	0.82	20.52	0.30		70	
	0.63	55.81	0.03		110	
	1.00	17.07	0.38		275	
11	17.77	296.11	0.39	0.43	135	11.50
	22.42	337.02	0.41		260	
	31.85	316.95	0.50		215	
	26.82	350.07	0.44		510	
14	14.35	232.78	0.39	0.43	505	8.00
	17.08	160.66	0.51		6080	
	20.79	329.89	0.40		900	
	18.06	256.91	0.42		200	
30	61.80	100.60	0.89	0.97	520	1.25
	249.20	181.09	1.07		760	
	234.88	228.88	1.01		1960	
	231.35	332.35	0.92		500	

* See footnote to Table II.

‡ See footnote to Table II.

cell suspensions interfered with each other. Thus, it seems fair to conclude that presence of free antigen plays no detectable role in this test system.

DISCUSSION

This article describes an inhibition method for antibodies produced by single hemolytic plaque-forming cells in vitro. The avidity of the antibodies produced by the PFC:s is reflected by the relative sensitivity of the plaques to inhibition by free antigen. Plaques formed by low avidity antibody require higher antigen

TABLE IV

Variation in the Avidity of the Antibody Response of Mice Immunized with 5000 μg of HSA into Each Hind Footpad: A Comparison between Serum Antibodies and Antibodies Released by PFC:s

Serum antibodies					Antibody-forming cells	
Day	ABC‡ at 1 μ/ml	ABC at 100 μ/ml	Factor of avidity		PFC:s in the regional lymph node	Agar plates giving 50% inhibition*
						μg antigen/ml
18	22.07	207.21	0.51	0.50	1380	3.19
	11.03	107.61	0.51		820	
	13.90	132.01	0.51		325	
	14.72	137.99	0.51		1200	
	15.15	200.29	0.44		375	
25	67.38	170.83	0.80	0.62	835	1.73
	90.65	557.17	0.61		2010	
	26.47	268.88	0.50		1490	
	66.73	686.63	0.49		1255	
	308.38	936.04	0.76		2350	
33	127.72	510.20	0.70	0.63	340	0.16
	48.95	659.43	0.44		430	
	110.58	669.12	0.61		205	
	137.92	325.31	0.81		1760	

* See footnote to Table II.

‡ Micrograms of HSA bound per milliliter of serum.

TABLE V

The Effect of Mixing Plaque Forming Cells from Animals Immunized with a High and a Low Dose of Antigen

Cell suspension	Plaques per plate*	Mixture of cell suspensions‡	Plaques per plate*	
			Expected	Obtained
5000 A§	5.5	5000 A + 50 A	667.5	689.0
5000 B	10.5	5000 B + 50 B	22.0	20.5
5000 C	59.0	5000 C + 50 C	67.5	72.5
50 A	662.0	5000 C + 50 A	721.0	796.0
50 B	11.5	5000 A + 50 C	14.0	20.0
50 C	8.5	5000 B + 50 C	19.0	13.0
		5000 A + 50 B	17.0	24.0
Total			1528.0	1635.5

* Mean value of two plates made from 0.1 ml of cell suspension.

‡ Mixtures were prepared by adding 0.1 ml of each cell suspension to the plates.

§ The figure indicates immunizing dose of BSA per foot pad and A, B, and C is identification of the cell donor. The test was performed on day 10 after immunization. A pool of suspensions 5000 A–C was inhibited 50% by 23.93 μg of BSA per ml of agar and a pool of suspensions 50 A–C by 0.77 μg of BSA per ml of agar.

concentrations for inhibition than high avidity plaques. Also in agreement with the concept of avidity is the finding that high avidity plaques are more resistant to inhibition by elution when free antigen is added to the plates after the antibodies are released from the PFC:s and attached to the target erythrocytes. Control experiments showed that free antigen was not transferred to the plates by the immune cell suspension in quantities that interfere with plaque formation.

It was found that both immunizing dose and time after immunization were important factors determining the avidity both at the serum level and when testing the PFC:s by inhibition. In essence, the significance of the findings described in this article is that it has been possible to show directly at the cellular level differences in avidity of antibody produced. The binding properties of the antibody synthetized vary with antigen dose and time after immunization, and the variations observed in the serum are not merely reflecting adsorption of antibody by the administered antigen. It would otherwise be quite feasible to postulate a preferential adsorption of the antibody with the highest affinity for the antigen, thus creating variations in the serum both as far as dose of antigen and time after immunization are concerned.

Some other advantages of the PFC inhibition method as compared to studies of affinity or avidity at the serum level can be noted: it is possible to determine at a given time the binding characteristics of the antibodies produced whereas determinations at the serum level reflect the cumulated antibody produced during a time period preceding the bleeding. It is also obvious that in experiments designed to study population kinetics in the induction of avidity changes by passive antibody or by paralyzing doses of antigen the present method allows the elimination of both antigen and antibody present in the circulation, since the PFC suspensions are washed prior to testing.

A disadvantage of the method is that PFC:s are relatively sensitive to variations in experimental conditions, and thus results obtained from different experiments may not be strictly comparable. The most reliable way to get information regarding avidity differences is to compare PFC populations by testing them the same day and under identical conditions. Most of the experiments described in this article have been conducted in this way.

The findings in this article are in agreement with earlier reports on the affinity of antibodies produced in vitro (26). These workers studied the antibody secreted into the supernatants of tissue-cultured lymph node cells by the use of incorporation of radioactive amino acids. Although a good deal of information could be obtained by that method, the method in some respects has the same limitations as determinations performed at the serum level; i.e., adsorption by antigen possibly being present in the immune cultures can not be excluded as definitely as in the present PFC system where the immune cells are separated from each other in the agar, and also where the short time needed to perform the test does not favor adsorption of antibodies produced.

Experiments on the influence of the number of haptens coupled to erythrocytes on hemolytic plaque formation have recently been reported (16). It was found that early in the immune response the plaque formation was more efficient with a high hapten density on the erythrocytes, whereas later in the immune response plaques were also formed with a lower hapten density. The differences observed in this case were most likely due to affinity changes.

SUMMARY

The hemolytic plaque formation of cells producing antibody against heterologous albumins was tested for sensitivity to specific inhibition by free antigen. The inhibition characteristics of plaques in this system were found to be a measure for the avidity of the antibody produced by the plaque-forming cells (PFC:s). High avidity–producing PFC:s were more sensitive to inhibition than low avidity PFC:s. Immunization with a high dose of antigen induced PFC:s that produced antibody with a lower avidity as compared to PFC:s from animals immunized with a low dose. The avidity was increased with time. Determinations of avidity at the serum level were also made, and the results were in agreement with the findings at the cellular level.

The present method made it possible to demonstrate differences in avidity of antibody at the level of the single antibody-forming cell. It may also constitute a useful tool for the analysis of the cellular events leading to the production of antibodies with varying affinities during the immune response.

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