EFFECTOR CELL BLOCKADE

A New Mechanism of Immune Hyporeactivity Induced by Multivalent Antigens*

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Of the many model antigens that have been used to elucidate the mechanisms of immunological nonresponsiveness, two groups have been especially popular. These are heterologous serum proteins (1) and highly multivalent, polymeric antigens such as pneumococcal polysaccharides (2) or polymerized flagellin (POL)[1] (3). In recent years haptens have been conjugated to such tolerogens, and hapten-specific tolerance at the level of the B lymphocyte has been measured. Though antigens from each group can cause B-cell nonresponsiveness under appropriate circumstances, the latter group exhibits some features which differentiate it from the former. These include strong immunogenicity in low (subtolerogenic) dosage, independence of help from T lymphocytes, a major IgM component to the primary response, and, in some cases, an adjuvantlike action. In fact, at least two antigens of this group which have been shown to be good in vitro B-cell tolerogens, namely bacterial lipopolysaccharide (4) and POL (3), actually possess the capacity to prevent the induction of B-cell tolerance by high doses of soluble serum proteins (5, 6). This suggests the possibility that the multivalent, T-cell independent antigens cause unresponsiveness through a different mechanism than that pertaining for soluble serum proteins.

In this paper, evidence is presented to show that highly multivalent antigens can specifically suppress immune responses at the level of the effector cell, namely the actual antibody-forming cell (AFC), by producing a substantial depression of antibody secretion rates of individual AFC. Thus in the case of thymus-independent antigens the possibility is raised that, under some experimental situations at least, the phenomenon previously believed to represent

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[1] *Abbreviations used in this paper:* AFC, antibody-forming cells; FCS, fetal calf serum; FGG, fowl gamma globulin; HGG, human gamma globulin; LPS, lipopolysaccharide; PFC, plaque-forming cells; POL, polymerized flagellin.

tolerance, in the sense of a failure of progenitors of AFC to become activated, may actually be an effector cell blockade.

Materials and Methods

Antigens.—POL was prepared from flagella of Salmonella adelaide (7). Fowl gamma globulin (FGG), prepared from serum by precipitation with sodium sulphate (8), human gamma globulin (HGG) (C.S.L., Parkville, Melbourne), and gelatine (Parke, Davis and Co., Sydney) were dinitrophenylated (DNP-FGG, DNP-HGG, and DNP-gel) using dinitrobenzene-sulphonic acid (Eastman Kodak Co., Rochester, N. Y.) as detailed elsewhere (9). The average conjugation ratios of the DNP-POL preparations used ranged from 1.0 to 2.9 mol of DNP per mole of monomeric flagellin (mol wt 40,000 daltons). The conjugation ratios of DNP-HGG preparations used were 4.5 and 20 mol of DNP per HGG. The DNP-gelatine had an average of 20 DNP groups per 100,000 dalton unit of gelatine. DNP-lysine (B.D.H., Melbourne, Australia) was conjugated to lipopolysaccharide (LPS) (Difco Laboratories, Inc., Detroit, Mich.) using a carbodiimide reagent, 1-cyclohexy-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulphonate (Aldrich Chemical Co., Milwaukee, Wis.). Complexes of FGG and POL were prepared by mixing POL with an antiserum raised in the domestic fowl against POL and washing the precipitate with saline (Schrader and Feldmann, manuscript in preparation). DNP-ε-amino-caproic acid (DNP-CAP) was obtained from B.D.H, Melbourne, Australia. DNP-DGL was obtained from Dr. David Katz.

Mice.—CBA/H/Wehi mice were used at 8–12 wk of age at first immunization.

Immunization.—Mice were immunized with DNP-POL 5 μg intraperitoneally (i.p.) and used from two to 7 days afterwards. For studies on the secondary response, mice were injected i.p. with alum-precipitated DNP-FGG, 500 μg, plus pertussis organisms (2×10^7 per mouse), and 1–3 mo later were boosted with an i.p. injection of 50 μg of soluble DNP-FGG.

Plaque-Forming Cell (PFC) Assay.—The hemolytic plaque technique of Cunningham and Szenberg (10) was used, suitably modified to detect hapten- (9) or protein-specific (11, 12) PFC. Unless otherwise stated, PFC assays were read after 60 min at 37°C, this period including a temperature equilibration time, since the assay mixtures were initially at 0°C and the assay chambers at room temperature. Routine assays were scored at 25-fold magnification with a colony microscope.

Preparation of Cell Suspensions.—Mice were killed by cervical dislocation, the spleens dissociated using tweezers, and the cell suspension washed once in cold Eisen's balanced salt solution (EBSS). Cells were suspended in Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) which was supplemented with 5% fetal calf serum (FCS) (C.S.L., Parkville, Melbourne), and buffered with Hepes (Calbiochem, San Diego, Calif.) (HEM).

Preincubation and Washing of Spleen Cells.—A preliminary assay was performed on the spleen cell suspension and the concentration of cells adjusted with HEM so that there were approximately 1,000 PFC/ml. Portions (0.5 ml) of the suspension (generally one spleen in 5-20 mls) were then incubated with varying amounts of the inhibiting antigen at 37°C or 0°C for varying periods. The cell suspensions were diluted with ice-cold HEM, washed twice by centrifugation through an FCS underlayer, resuspended, and assayed for PFC.

Micromanipulation Studies.—An open modification of the hemolytic plaque technique was used, in conjunction with micromanipulation techniques as described elsewhere (13, 14). Briefly, a starting population of PFC was generated by placing immunized spleen cells in a plaque-revealing mixture in a series of large microdroplets (approximately 1 μl) on glass slides under paraffin oil. In some cases, the cell suspension had been preincubated for 30 min at 37° with multivalent antigen, and washed twice, before generating plaques ("preinhibited PFC"). Microscope and micromanipulator were at 37°C in a plastic incubator for some experiments and at room temperature (approximately 20°C) for others. In experiments where it was

desired to place PFC into ^{125}I-labeled antiglobulin at 0°C, slides bearing the appropriate microdrops and all necessary media as well as the covering paraffin, were held on packed ice, except for the actual few minutes taken for the micromanipulation step, which was performed with the microscope at room temperature. When a hemolytic plaque was identified with only a single, central PFC, this cell was removed by micromanipulation, washed twice in droplets of clean medium, and processed for further study.

Detection of Cell Surface Ig on Single PFC by Quantitative Radioautography.—The technique was slightly modified from that described previously (14). Individual PFC that had come from preinhibited or normal plaques were washed twice and held at 0° or 37°C, usually for 30 min. with an ^{125}I-labeled antiglobulin. The reagent used was a strong, polyvalent rabbit antimouse Ig immunoglobulin previously described in detail (R19NS), (15). It was used at a concentration of 0.1 μg/ml, with 0.5–0.6 [^{125}I]atoms/mol of Ig. After two further washes by micromanipulation, the single cells were manipulated onto premarked spots on gelatin-coated slides, processed for radioautography with Kodak NTB2 emulsion, exposed for 3 days, developed, stained with Giemsa, and the resultant grains counted at × 1,250 magnification.

RESULTS

Inhibition of Plaque-Formation by Prior Incubation of Spleen Cells with DNP-POL.—Most experiments were performed with AFC that made anti-DNP antibody of the IgM class and that were contained in spleen cell suspensions from mice injected 3–5 days previously with DNP-POL (5 μg i.p.). If before assay, the spleen cells were incubated at 37°C with DNP-POL and then washed thoroughly, the number and quality of plaques were reduced in comparison with the results of assays of control aliquots incubated in medium alone and washed in parallel. The degree of inhibition varied with the concentration of DNP-POL, (Fig. 1) or incubation time (Table I). 1-h incubation in medium alone caused a slight but definite loss in PFC numbers as observed by

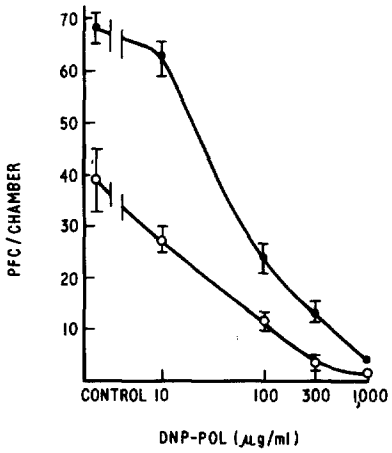

FIG. 1. The effect of varying the dose of DNP-POL in the preincubation mixture. Portions of spleen cell suspensions containing anti-DNP-AFC were incubated at 37°C for 30 min with varying concentrations of DNP-POL. After washing twice, the cells were suspended in 1 ml HEM. Assays were made in two cohorts, one being read after 60 min incubation (●—●) the other after 30 min (○—○).

Hiramoto et al. (16), but 60 min in inhibitor caused an almost total elimination of PFC. With 30 min of preincubation in medium alone at 37°C, the nonspecific loss of PFC was essentially eliminated, and the specific inhibition was somewhat less marked. This shorter incubation time was used for most experiments.

Another control, both for nonspecific deterioration of PFC and for nonspecific toxicity of multivalent conjugates, was to include in the cell suspension a second population of PFC, directed against an irrelevant antigen. Table II illustrates

TABLE I

The Effect of Variation of the Time of Preincubation with DNP-POL

Pretreatment		Anti-DNP PFC/chamber	
100 μg DNP-POL	Incubation time	Numbers‡	%
	min		
+	60	4, 9	5
−	60	99, 99	83
+	30	26, 40	27
−	30	129, 115	100
−	—*	134, 121	104

A spleen cell suspension prepared from two CBA mice injected 3 days before with DNP-POL, was diluted with HEM so as to contain approximately 2,500 AFC/ml. Portions of 0.3 ml were incubated with or without DNP-POL at 37°C for either 60 min or 30 min. All groups of cells were washed twice in 10 ml of cold EBSS and resuspended to 0.6 ml in HEM. Duplicate 0.1-ml portions were assayed for anti-DNP-AFC along with aliquots from the original suspension* which had not been incubated or washed. Results are shown as the actual PFC numbers per chamber,‡ or the mean PFC/chamber expressed as the percentage of the mean PFC in the assay of the control group incubated with medium alone for 30 min.

TABLE II

Specificity of the Effect of Preincubation with DNP-POL

Concentration of DNP-POL*	% PFC/chamber	
	Anti-DNP	Anti-FGG
μg/ml		
400	40 ± 1	104 ± 2
100	45 ± 9	99 ± 4
10	90 ± 4	97 ± 2
1	93 ± 1	101 ± 2
—	100	100

Spleen cells from mice injected 4 days previously with DNP-POL, 5 μg and from other mice injected 4 days previously with FGG, 100 μg, plus POL 10 μg, as an adjuvant (manuscript in preparation), were assayed for anti-DNP and anti-FGG-AFC and then diluted and mixed so that 1 ml of the final suspension contained approximately 1,000 AFC of each specificity. Duplicate portions ot 0.5 ml were incubated for 30 min at 37°C with varying concentrations of DNP-POL*, and washed twice before duplicate assays were set up for AFC of each specificity. Results are pooled from four experiments and are normalized, with the AFC counts in control portions being taken as 100%.

the results of four typical experiments which showed that, while DNP-POL inhibited anti-DNP plaques, it had no effect on anti-FGG plaques.

Tables I and II present counts of all plaques detected, regardless of size or morphologic characteristics. However, simple observation showed that those plaques remaining in DNP-SRC monolayers after pretreatment of the cell suspension with DNP-POL were consistently smaller and more turbid than control plaques. Thus, the inhibition phenomenon at the single PFC level appeared not to be an "all or none" effect. Rather, the changed appearance of residual plaques suggested that all PFC showed a reduced rate of antibody secretion. The following experiments supported this suggestion.

Time of Appearance of Normal and Preinhibited Plaques.—Fig. 2 shows the effect of varying the time of incubation of the assay chambers. Clearly, preinhibited PFC showed a delayed time of plaque appearance. The lower curve in Fig. 1 represents the results of readings at 30 min of assays of aliquots preinhibited with varying doses of DNP-POL and demonstrates the same phenomenon.

Effects of Suboptimal Plaque-Revealing Conditions.—The results displayed in Table III show that decreasing the efficiency of the plaque-revealing system by limiting the amount of complement or increasing the DNP-SRC concentration increased the difference between PFC numbers in aliquots of DNP-POL-treated and control suspensions. These results suggest that PFC are not being deleted, for example by complement-mediated lysis, but rather that their rate of effective antigen secretion is being diminished. Obviously, at cell secretion rates below a threshold determined by the sensitivity of the plaque assay, AFC will be no longer detectable at all as PFC.

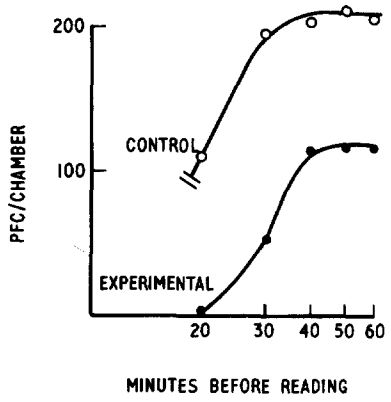

FIG. 2. Time of appearance of PFC in assays of DNP-POL treated anti-DNP AFC suspensions. Portions of a spleen-cell suspension were incubated in the presence or absence of DNP-POL (100 μg/ml) for 30 min at 37°C and then washed twice. Ten assays were set up on each portion and read after varying periods of incubation at 37°C, each point graphed representing the mean of two assays.

TABLE III

The Effect on the Degree of Inhibition of PFC Numbers of Suboptimal Assay Conditions

Conditions of Assay	Controls*	Experimental‡	% inhibition§
Standard	100	82	18
Read at 20 min‖	65	16	75
66% C'¶	90	68	24
33% C'	45	9	80
16% C'	0	0	—
1.8% DNP-SRC	86	45	48

Spleen cells containing anti-DNP-AFC were incubated with either 100 μg/ml of a preparation of DNP-POL‡, or HEM alone*, for 30 min at 37°C and washed twice. Duplicate assays were made of the control and experimental aliquots under various assay conditions. Thus the standard 60-min incubation time was reduced to 20 min,‖, the complement (C') concentration was reduced to the indicated percentage of the standard amount,¶, and the concentration of DNP-SRC was increased from the standard 1.25% up to 1.8%. Figures in ‡,* represent mean PFC/chamber. The calculated percentage inhibition § is shown for each set of assay conditions. The batch of DNP-POL differed from that used in earlier experiments where the inhibition under standard conditions was greater but is used to show more clearly the effect of varying assay conditions.

The Effect of Assaying Together Pretreated and Control Suspensions.—It was conceivable that the inhibitory effect seen in these experiments was due to antigen that had been carried over from the preincubation stage into the assay and which diffused out into the assay mixture, thus inhibiting plaque formation by competing for secreted antibody with the DNP-SRC (17). Therefore, aliquots pretreated with DNP-POL were mixed with aliquots preincubated with medium alone, and the mixture assayed for anti-DNP-AFC. Table IV demonstrates that the presence of cells from the "inhibited" cell suspension had no effect on the emergence of plaques due to the AFC from the control suspension. It was also noted that plaque morphology was as expected from this; in assays on the mixture, two populations of plaques could be distinguished, one of large clear plaques, similar to those in assays of control aliquots, the other of small indistinct plaques, similar to those seen in assays of the pretreated aliquot. This indicated that if antigen-carry over is involved, then this antigen must be bound to the individual PFC that are inhibited.

The Effect of Binding DNP-POL onto Cell Suspensions at 0°C.—Spleen cell suspensions were exposed to DNP-POL at 0°C for 30 min, were washed, and then held at 37°C for varying periods before being assayed. Table V shows that exposure to DNP-POL at 0°C followed by washing resulted in inhibition of anti-DNP-PFC, provided that the cells had been incubated at 37°C for 30 min before entering the assay. This experiment suggested that DNP-POL was binding to the cell suspension since it is difficult to envisage how contact with AFC at 0°C could otherwise affect the subsequent ability to form plaques. Taken together with the previous mixing experiment which showed that only

TABLE IV

The Effect of Mixing Pretreated with Control Cells on Expected PFC Number

Pretreatment*	PFC/chamber	
	30 m	60 m
DNP-POL‡	44 ± 7	58 ± 11
Control§	101 ± 6	102 ± 17
(DNP-POL + control)‖	77 ± 1 (73) (62)	92 ± 14 (80) (80)
½ (DNP-POL)¶	22 ± 2	32 ± 6
½ (Control)	40 ± 3	48 ± 3

* Portions (0.5 ml) of a pooled spleen suspension from DNP-POL immunized mice, were incubated for 30 min at 37°C with DNP-POL, (100 μg/ml)‡, or medium alone.§ Portions were washed twice and resuspended in 4 ml of HEM. Anti-DNP-PFC assays were made of these suspensions, and also of a mixture‖ containing 1 ml of each of‡ and§. Twofold dilutions of the experimental‡ and control§ suspensions were also assayed.¶ Figures shown in parentheses represent expected results of the assay of the mixture, the upper figures representing the mean of‡ and§ values, the lower figure the sum of the results of assays on the diluted suspensions.¶

TABLE V

The Binding of DNP-POL to Spleen Cell Suspensions at 0°C

Exposure to DNP-POL*	Incubation after washing	PFC/chamber	
		Experimental	Control‡
37°C	—	126 ± 3 (53)§	237 ± 10
0°C	—	258 ± 22 (118)	218 ± 22
0°C	30 min	106 ± 6 (46)	232 ± 20
0°C	10 min	219 ± 24 (98)	224 ± 11

* Portions of spleen cells containing anti-DNP-AFC were exposed to DNP-POL, 100 μg/ml for 30 min at either 37°C or 0°C. After washing the spleen cells were assayed immediately or were incubated at 37°C for 30 or 10 min before assay. Control portions‡ were treated in parallel except that DNP-POL was not present. The figures in parentheses,§ represent experimental values expressed as a percentage of the respective control.

those AFC in the pretreated population were affected, the inference is that the relevant binding must be to the AFC themselves. These results show that the binding of antigen to the AFC could occur at 0°C, while the AFC were not actively secreting antibody. Finally this experiment suggests that for the inhibitory effect to become manifest, the cell had to be at 37°C for a certain period.

The Effect on IgG-Producing AFC.—An additional finding was that IgG-secreting AFC could be inhibited by a similar preincubation with DNP-POL (Table VI). There was no detectable change in the susceptibility to interfer-

ence with plaque formation of AFC tested on various days up to the 12th day of secondary response.

The Importance of the Multivalent Nature of the Antigen in Effector Cell Blockade.—We next studied the capacity of a variety of DNP conjugates to act as specific inhibitors of AFC (Table VII). Highly multivalent molecules such

TABLE VI

The Effect on IgG-AFC of Preincubation with DNP-POL

Day after boosting	Preincubation with DNP-POL	Indirect anti-DNP-PFC/chamber	
		Number	%*
	μg/ml		
3	100	46 ± 10	52
	—	90 ± 1	100
11	100	58 ± 3	66
	10	103 ± 4	96
	—	107 ± 6	100

Spleen cell suspensions were prepared from mice injected 1 mo previously with alum-precipitated DNP-FGG plus 2×10^7 pertussis organisms and boosted 3 days previously with 100 μg DNP-FGG. Portions were incubated at 37°C for 30 min with DNP-POL as indicated and washed twice. In the second experiment mice were killed and spleen cell suspensions prepared 11 days after boosting with DNP-FGG. Column* represents PFC numbers expressed as a percentage of values in assays of portions incubated with medium alone.

TABLE VII

The Efficacy of Various Multivalent DNP-Conjugates in Effector Cell Blockade

DNP-conjugate		Molarity of DNP	PFC/chamber	
			Anti-DNP	Anti-FGG
	μg/ml			
DNP-CAP		10^{-4}	94	ND
		10^{-3}	100	ND
$DNP_{4.5}HGG$	100	3×10^{-6}	98	105
"	1,000	3×10^{-5}	80	90
DNP_{20}-HGG	10	1.3×10^{-6}	77	102
"	25	3×10^{-6}	44	99
"	100	1.3×10^{-5}	40	108
DNP-D-GL	0.1	7.4×10^{-8}	105	100
"	1	7.4×10^{-7}	57	109
"	10	7.4×10^{-6}	38	105
DNP-LPS	25	—*	45	ND

Spleen cell suspensions containing approximately 1,000 anti-DNP-AFC per ml were incubated with the indicated amounts of various DNP-conjugates for 30 min at 37°C and washed as in previous experiments. Assays were set up in duplicate or quadruplicate and read after 60 min. In some cases FGG-specific AFC were included in the test suspensions and were assayed for in parallel. Results are expressed as a percentage of the mean number of PFC in control portions incubated in medium alone. * Not measured. ND, not done.

as DNP-LPS and DNP_{37}-DGL, a random copolymer of D-glutamic acid and D-lysine with a mean mol wt of 50,000 (18, 19) were efficient inhibitors. DNP lysine (data not shown) or DNP-ε-amino-caproic acid were not inhibitors, while lightly substituted $DNP_{4.5}$HGG was only minimally inhibitory in contrast to DNP_{20}-HGG. It was clear that valency was a more important variable than hapten molarity.

Specific Inhibition of AFC of other Specificities by Multivalent Antigen.—Similar effects could be demonstrated with AFC of different specificities. Thus anti-FGG AFC could be inhibited by prior incubation with a multivalent antigen-antibody complex formed by the interaction of fowl anti-POL antibodies with POL (FGG-POL), but not by soluble FGG (Table VIII). Like-

TABLE VIII

Blockade of PFC of Differing Specificities

Preincubation with:		PFC/chamber	
		Anti-POL	Anti-FGG
	μg/ml		
OXMON	100	104	
POL	100	42	
"	10	98	
FGG	300		101
"	50		106
FGG-POL	100		53
"	10		102

Spleen cell suspensions containing AFC of appropriate specificities, were incubated with the corresponding antigen as indicated for 30 min at 37°C and washed as before. Assays were set up in duplicate or quadruplicate and read at 60 min. Results represent mean PFC/chamber expressed as a percentage of values in assays on control portions incubated with medium alone.

wise, AFC directed against flagellar antigens were inhibited by POL but not by the monomeric form of flagellin, here oxidized to a form in which repolymerization does not occur (OXMON) (20) (Table VIII).

The Importance of the Substitution Ratio of the DNP-POL.—Previous work using DNP-POL as a tolerogen in vitro has stressed the importance of a high substitution rate of DNP groups in the induction of DNP-specific tolerance (9). Therefore it was of interest to examine this variable in the present system. Table IX shows that $DNP_{2.9}$-POL was more effective than $DNP_{1.1}$-POL in inhibiting formation of plaques by anti DNP-AFC. With $DNP_{1.1}$-POL some inhibition was detectable with preincubation of AFC at 100 μg/ml. Using this preparation of $DNP_{2.9}$-POL, inhibition was marked at 100 μg/ml and detectable at 10 μg/ml, especially in assays read at 30 min (Table IX).

In Vivo Inhibition of the Expression of PFC Capacity.—Mice which had been injected with DNP-POL, 5 μg i.p., 4 days previously were injected with various

DNP conjugates intravenously. 4 h later the mice were killed and the spleens assayed for PFC. Table X shows that PFC numbers were decreased in animals injected with suitable doses of DNP conjugates. It was striking that, as in the in vitro experiments, the plaques formed by those AFC escaping total inhibition were faint and small.

TABLE IX

*The Effect of Incubation of PFC with DNP-POL of Different Substitution Ratios**

DNP per MON	Concentration DNP-POL	Molarity DNP	PFC/chamber			
			30 min		60 min	
	μg/ml			%		%
—	—	—	50 ± 8§	100‖	68 ± 2§	100‖
1.1	100	2.8×10^{-6}	41 ± 2	82	51 ± 4	75
	10	2.8×10^{-7}	38 ± 1	76	73 ± 6	107
2.9	100	7.2×10^{-6}	5 ± 2	10	29 ± 9	43
	10	7.2×10^{-7}	27 ± 3	54	52 ± 1	77

* Portions of anti-DNP-AFC were incubated for 30 min at 37°C with DNP-POL of two substitution ratios‡ at the concentrations indicated. After washing twice, PFC assays were set up, one cohort being read at 30 min, the other at 60 min. Results are expressed as mean PFC/chamber,§ and also as a percentage of the control value.‖

TABLE X

In Vivo Inhibition of Anti-DNP-PFC

Pretreatment of mice	Anti-DNP PFC/Spleen	Plaque morphology
—	6,050 ± 600	Large, clear
DNP-Gelatine 10 mg	4,350 ± 1,900	Small, turbid
DNP-DGL 500 μg	2,300 ± 230	Very small, very turbid

CBA mice which had been injected 3 days previously with DNP-POL were injected intravenously in groups of three with the indicated amounts of two DNP-conjugates. 4 h later mice were killed and the spleens assayed individually for anti-DNP-PFC. Results are expressed as the mean AFC/spleen ± the SE of the mean.

Antibody Does not Accumulate on the Surface of Inhibited AFC.—The two explanations for the interference with plaque formation by AFC seen in this study are (*a*) that antigen is adherent to the AFC where it traps antibody as it is secreted, thus preventing diffusion out into the plaque-revealing monolayer and (*b*) that there is an actual diminution of antibody secretion by the cell. Therefore a micromanipulation technique was employed to determine directly the amount of immunoglobulin on the surface of individual AFC from inhibited and normal populations. Preliminary experiments showed that inhibition could be satisfactorily demonstrated by PFC assays in the microdrop system (Fig. 3). Thus with appropriate concentrations of DNP-SRC and complement, a definite inhibitory effect in terms of rate of plaque appearance and final number could

be shown, but by 20 min some inhibited AFC were revealed and could be selected by micromanipulation. After 20 min AFC were taken from definite plaques, irrespective of plaque size, in the order in which they were encountered on the slide, one AFC being taken alternately from the control and experimental groups of drops. Individual AFC were held at 0°C or 37°C for 20–30 min in 0.1 μg/ml of [^{125}I]RAMG, were washed twice, and then spread individually for radioautography. Table XI shows the resultant grain counts. As expected from previous work (14), there was a considerable scatter in grain

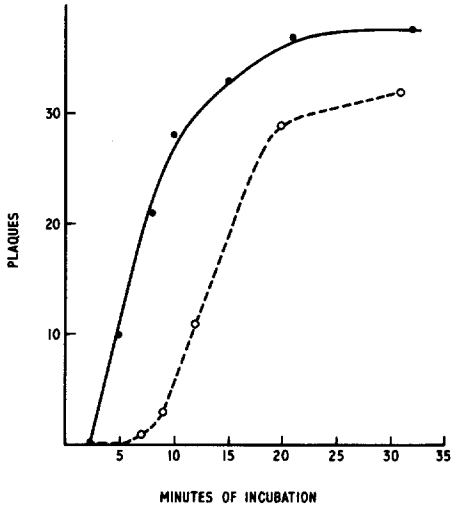

FIG. 3. Time of appearance of plaques made by control or preinhibited AFC in microdroplets. Anti-DNP-AFC were incubated at 37°C for 30 min with HEM alone or with DNP-POL, 100 μg/ml and washed as before. 20 microdroplets assays were then set-up for control (●—●) and experimental (○---○) aliquots and the plaque numbers counted in each at succeeding intervals.

TABLE XI

Radioautographic Studies on Single Cells to Detect Surface-Bound Ig on Normal and Preinhibited PFC using [^{125}I]Antiglobulin

Nature of PFC	Temperature during antiglobulin binding	No. of PFC studied	Grain count		*P* value (*t* test)
			Mean ± SE	Range	
	°C				
Preinhibited	37	17	28.1 ± 8.4	0–124	NS
Control	37	19	23.7 ± 5.4	0–74	NS
Preinhibited	0	19	29.0 ± 6.8	0–105	NS
Control	0	16	19.5 ± 4.1	0–61	NS

Preinhibited or control cells were placed in plaque-revealing microdrops under paraffin oil. Control PFC were removed by micromanipulation, washed, and placed in 0.1 μg/ml [^{125}I]-antiglobulin. After binding for 20–30 min in the 37° group, or 30 min in the 0° group, the cells were processed for radioautography.

counts in the control population, reflecting variation in accessible surface Ig amongst PFC. A similar variation was encountered amongst preinhibited PFC, and there was no significant difference between the populations in terms of labelling with [^{125}I]RAMG. Thus there was no evidence that PFC from the inhibited population had on the average accumulated greater amounts of antibody on their surface. This conclusion is valid only if the attached antigen does not sterically hinder access of [^{125}I]RAMG to cell surface Ig.

Blockade Studies on Single, Normal PFC Using Micromanipulation Techniques.—We wished to show directly that a given PFC could be subjected to blockade, and could thereby be forced to reduce its antibody secretion rate, without having its viability impaired. Micromanipulation offered this opportunity. Single, anti-DNP-PFC were taken from the center of normal hemolytic plaques within the first 20 min of incubation of DNP-POL immunized spleen cells in plaque-revealing droplets. Each single cell was washed twice, and placed in one of two coded droplets, the one containing HEM-FCS alone, the other DNP-POL, usually at a concentration of 100 μg/ml. After 30 min at 37°C, each cell was washed twice and micromanipulated into a plaque-revealing drop. With the aid of an eye-piece vernier, the area of hemolysis appearing over the next 30 min at 37°C was repeatedly measured. It was noted that, after a latent period, the area of hemolysis was directly proportional to incubation time. At the conclusion of each experiment, the results were decoded. The results of a pool of experiments are given in Table XII. It is shown that plaque size was inhibited by an average of 68% through effector cell blockade, the effect being statistically significant.

In a separate study, involving 22 PFC, cells were taken in pairs, approximately matched for plaque diameter at the first selection stage, and consciously assigned to blockading or control droplets in such a manner that the PFC forming the bigger plaque always went into inhibitor, and that forming the smaller into medium alone. After only 15 min at 37°C, the cells were assessed for hemolytic capacity, and, even with this intentional bias introduced, a 62% inhibition of plaque size through blockade was noted.

TABLE XII

Diminution in Antibody Secretion Rate Resulting from Blockade of Single PFC by DNP-POL

Nature of inhibitor	No. of PFC studied	Area of hemolysis at 20 min ($\mu^2 \times 10^{-4}$)	*P* value (*t* test)
		Mean ± *SE*	
Medium only	11	13 ± 3.9	$0.02 < P < 0.05$
100 μg/ml DNP-POL	12	4.2 ± 0.27	

Single PFC were taken from anti-DNP plaques by micromanipulation, blindly assigned to control or inhibitor microdrops, and then assessed for antibody-secreting rate by measuring plaque diameter at successive time intervals. The area of hemolysis was plotted against time, and resulted in a straight line. The mean area of hemolysis at 20 min is shown above.

DISCUSSION

This study shows that the appearance of PFC can be specifically inhibited by prior contact of the AFC with a multivalent form of the antigen. The magnitude of this inhibition was inversely proportional to the sensitivity of the assay, and micromanipulation studies showed that inhibition involved a decreased rate of antibody secretion by individual AFC. The mechanism of the blockade effect is not clear. The present experiments, especially those where the spleen cells are exposed to DNP-POL at 0°C and then washed before warming to 37°C (Table V) imply that the antigen is binding to the spleen cells. Since the effect is antigen-specific such binding is likely to be to the specific immunoglobulin receptors. In this connection the reported increasing sparsity of immunoglobulin receptors on more mature AFC (14) is of interest since the inhibition of IgG-AFC 11 days after boosting was still potent (Table VI). It is possible that the increased affinity of the few receptors of more differentiated AFC appearing later in the response, may account for their suppression. In any case, the effect does not depend on the killing of PFC, as the single cell experiments show clearly.

The present experiments demonstrate that multivalent thymus-independent antigens can inhibit the expression of an immune response at the level of the final effector cell, a cell usually thought of as autonomous and not subject to regulation by antigen. The effect is sufficiently profound in some cases, particularly where plaque revelation is suboptimal, to raise the issue whether certain phenomena regarded as tolerance might represent no more than a blockade of the AFC by persisting antigen. Baker et al. (21) investigated tolerance to pneumococcal polysaccharide in vivo, and noted an effect which we believe to be due to the phenomenon described in this paper. As antigen doses were raised from optimally immunogenic to subtolerogenic, they showed a decrease in secretion rate by individual AFC. Similarly, Table X shows that our phenomenon can operate in vivo, suggesting a parallel to the work of Mitchell and Humphrey (22), who blocked anti-DNP responses by injecting, early in the response, DNP-pneumococcal polysaccharide. Furthermore, in other examples of "tolerance" to multivalent, persistent antigens (23, 24), increased numbers of antigen-binding cells have been noted. These could represent blocked AFC.

In certain models, the tolerant state is rapidly reversed on transfer of cell populations to an antigen-free environment such as a lethally X-irradiated host (2, 25). This probably reflects the shedding of cell-bound antigen, in some way responsible for holding the cells "tolerant." Some multivalent antigens like levan which do not demonstrate such reversibility (26), may have a higher avidity for the surface of immunocompetent cells and not be shed, as discussed by Kotlarski et al. (27). While this paper has shown that the actual AFC can be blocked by multivalent antigen, similar mechanisms could operate at the level of the B-cell progenitor of AFC. In that case, B cells with excess attached

multivalent antigen might be inhibited from division and differentiation. This could be regarded as a true central tolerance of B cells but differing from that caused by oligovalent, T-cell dependent antigens (6). It may be significant that only in the case of B-cell tolerance to thymus-dependent antigens is there an actual decrease in numbers of antigen-binding cells (28).

It is possible that soluble complexes of antigen and antibody formed in slight antigen excess may induce effector cell blockade. These complexes are powerful suppressors of humoral responses (29, 30), and may be involved in blocking the cytotoxic killing of tumor cells by lymphocytes (31). Blocking effects at the T-cell level may represent a similar phenomenon to that described here at the level of the AFC. Finally, if highly multivalent, poorly degradable antigens readily blockade T cells, their inefficiency in producing delayed hypersensitivity or IgG antibody production, that requires T-cell help (32) is explained.

The possibility of a regulatory role for multivalent antigens and immune complexes at the level of the actual effector cells has many implications for the study of immune regulation. The mechanism of the phenomenon at cell biologic and molecular levels presents a challenge for the immediate future.

SUMMARY

This study describes the effects of incubating antibody-forming cells (AFC), either as mass cell suspensions, or as single AFC in microdroplets, with antigens against which the cells display specificity. Most of the work was done with hapten-specific anti-DNP-AFC, but AFC with specificity against flagellar antigens or fowl gamma globulin (FGG) were also included. It was noted that 30-min incubation of AFC with highly multivalent forms of antigen caused a substantial partial suppression of the antibody-forming performance of the AFC as measured by a hemolytic plaque test. Thus, when cell suspensions containing anti-DNP plaque-forming cells (PFC), were incubated for 30 min at 37°C with 100 μg of DNP-polymerized flagellin (DNP-POL), the number of plaques appearing after washing of the cells and placing them in plaque-revealing erythrocyte monolayers was reduced to 50% or less compared with the number of plaques observed with control portions preincubated with medium alone. Preincubation with DNP-lysine, with oligovalent DNP-protein conjugates, or with irrelevant antigens produced no such inhibition. Studies where preinhibited PFC suspensions were mixed with control suspensions before assay showed that a nonspecific carryover of antigen into the assay system was not involved. The inhibitory effect could also be initiated by holding cells at 0°C with DNP-POL, but in that case, inhibition only became manifest after cells were incubated for 30 min at 37°C before being placed in plaque-revealing monolayers. This suggested that inhibition was initiated by adsorption of multivalent antigen onto PFC-surface Ig, but required some active process before secretion actually slowed down. The effect was dose- and time-dependent, antigen-specific, and generalized for all antigens studied.

As well as yielding reduced plaque numbers, the preinhibited cells also gave smaller, more turbid plaques, suggesting a reduction in antibody-forming rate by each PFC rather than the elimination of PFC. Consistent with this suggestion was the observation that the degree of inhibition of plaque formation could be increased by decreasing the sensitivity of the assay so that only AFC secreting at high rates were detected. A micromanipulation study, where single PFC were subjected to inhibition, and were then tested for the rate at which they could cause hemolysis, showed a 68% inhibition of mean secretory rate.

Micromanipulation studies were performed to test the amount of cell surface-associated Ig on control and preinhibited PFC. For this, single PFC were held with [^{125}I]antiglobulin and quantitative radioautography was performed. No significant difference emerged, suggesting that retention of secreted Ig on cell-attached antigen was not the cause of inhibition.

The results are discussed in the framework of tolerance models and blocking effects at the T-cell level by antigen-antibody complexes. The name effector cell blockade is suggested in the belief that the phenomenon may be a general one applying to both T and B cells.

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