MECHANISM OF EFFECTOR-CELL BLOCKADE

I. Antigen-induced Suppression of Ig Synthesis in a Hybridoma Cell Line, and Correlation with Cell-associated Antigen

By ANDREW W. BOYD AND JOHN W. SCHRADER

From the Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Victoria 3050, Australia

Previous work from this laboratory (1, 2) has characterized the phenomenon of effector-cell blockade (ECB),[1] in which interaction of an antibody-forming cell with specific antigen results in inhibition of antibody secretion. Other examples of ECB have been reported by Baker et al. (3) and by Klaus and Humphrey (4).

Multivalency of the antigen, that is, high hapten:carrier ratio was found to be critical (1, 2, 5, 6). Furthermore, the removal of cell-bound antigen resulted in reversal of the blockade (2), suggesting that inhibition of antibody secretion is mediated by persistence of antigen on the cell membrane. Thus, ECB is of interest not only as an example of immune regulation but also as a potential example of membrane-immunoglobulin-mediated transmission of regulatory signals.

The particular advantage of ECB as a model for the study of at least one type of Ig-receptor-mediated signaling is that the effects of antigen binding are detectable after only 20 min. Furthermore, changes can be studied at the level of the single cell, where other cell-cell interactions are absent (1), and involve the major secretory product of the cell, specific antibody. ECB is detectable not only in normal antibody-forming cells; but also in their neoplastic counterparts, myeloma cells (7–10).

Cloned cell lines such as myelomas offer significant advantages in elucidating the molecular mechanisms underlying this phenomenon because they can be grown in quantity as a relatively homogeneous population by standard tissue culture methods. Abbas and Klaus (7, 8) and Abbas (10) have used the IgA-secreting, dinitrophenol (DNP)-binding myeloma MOPC 315 to show that secretion of IgA is inhibited by suitable DNP-conjugated proteins or by antigen-antibody complexes. In our study, we describe the production and characterization of a hybridoma cell line that was designed to facilitate a parallel study of the biochemical and membrane-receptor changes occurring in ECB.

[1] *Abbreviations used in this paper:* AFC, antibody-forming cells; BSA, bovine serum albumin; DME, Dulbecco's modified Eagle's medium; DNP, dinitrophenol; ECB, effector-cell blockade; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FLU, fluorescein-(ated); FluIgM-1, ascites fluid from tumors that contained IgM; GEL, gelatin; HAT, hypoxanthine-aminopterin-thioguanine; HGG, human gamma globulin; HT medium, hypoxanthine-thioguanine-supplemented DME; NET buffer, 150 mM/liter NaCl, 5 mM/liter EDTA, 50 mM/liter Tris, and 0.2% sodium azide (wt:vol); NIP, mononitroiodophenyl; NP40, Nonidet P-40; PBS, phosphate-buffered saline; PFC, plaque-forming cells; POL, polymerized flagellin; PVC, polyvinyl chloride; RIA, solid-phase radioimmunoassay; SRBC, sheep erythrocytes; TCA, trichloroacetic acid.

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The cell line we describe secretes IgM antibody specific for the hapten fluorescein (FLU) that through its fluorescence properties offers a simple means of directly monitoring the fate of antigen bound to the cell surface. We document here the susceptibility of the cell line to ECB, as measured both by the inhibition of hemolytic plaque formation and by the specific reduction of Ig secretion and synthesis. Parallel observations on the fate of cell-bound antigen are described.

Materials and Methods

Antigens. FLU conjugates were prepared by coupling fluorescein isothiocyanate (FITC; Molecular Probes, Rockville, Minn.) to proteins in bicarbonate buffer (pH 9) (11). The reagent most extensively used in this study was FLU_{20} gelatin (GEL) ($FLU_{20}GEL$), prepared by coupling 20 mg of FITC per 100 mg of gelatin (Parke, Davis & Co., Sydney, Australia). This yielded a conjugate having 20 FLU groups/10^5 daltons of gelatin.

FLU-bovine serum albumin (BSA) (FLU_{10} BSA) was prepared in a similar manner using crystalline BSA (Armour Pharmaceutical Co., Eastbourne, England). The other FLU conjugates, generously supplied by B. L. Pike, Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Victoria, Australia, were prepared in the same fashion.

Animals. BALB/c mice were used as the source of normal antibody-forming cells in the hybridoma fusions. Subsequently, cloned hybridoma lines were injected intraperitoneally (10^7 cells/mouse) to establish ascitic tumors. The tumor was passaged in these mice, and resultant ascites fluid that contained hybridoma IgM (FluIgM-1), was harvested for use in immunoprecipitation assays described.

Tissue Culture Conditions. Cells were cultured in Dulbecco's modified Eagle's medium (DME) with 10% fetal calf serum (FCS) in a 10% CO_2 incubator. Both hybridoma cells and NS-1 (the parent myeloma line) were cultured under identical conditions. Cells were harvested late in the log phase of growth at a density of $\sim 5 \times 10^5$/ml, at which stage viability was high (>85%).

Hybridoma Production. BALB/c mice were immunized intraperitoneally with 10 μg/ml of FLU-conjugated, polymerized flagellin (POL) (FLU-POL) 3 d before fusion. Previous experience with hemolytic plaque assays showed that IgM-secreting antibody-forming cells were most numerous at this stage. These mice were killed by cervical dislocation and the spleens removed. Spleen cells were carefully teased out with forceps, yielding a high viability (>90% after washing in fresh medium) population of spleen cells in serum-free DME. Fusion of 1.4×10^8 spleen cells and 1.4×10^7 hypoxanthine-aminopterin-thioguanine (HAT)-sensitive, nonsecreting myeloma cells (NS-1) was performed, using the technique of Köhler and Milstein (12).

The fusion products were then incubated in DME-15% FCS for 24 h before aliquoting into two 24-well Linbro trays (No. 76-033-05; Linbro Chemical Co., Hamden, Conn.) with 2×10^6 cells per well, and 64 of the 96 wells of a Linbro No. 76-013-05 tray with 5×10^5 cells/well, which contained 1 ml and 0.2 ml of HAT medium, respectively.

The wells were fed every 48 h for 2 wk with HAT medium. At the end of this period feeding was continued with hypoxanthine-thioguanine-supplemented DME (HT medium), and wells were examined for growth. Supernates were removed from wells showing proliferation and were tested for the presence of Ig by solid-phase radioimmunoassay (RIA) on $FLU_{10}BSA$-coated plates (see below), and by hemolytic plaque testing. The cells in wells that contained the most antibody were cloned by limiting-dilution with 10% positive wells used as the end point.

Antisera. Rabbit anti-FluIgM-1 was obtained by immunizing rabbits with 500 μg of purified hybridoma protein in complete Freund's adjuvant every 4 wk. The hybridoma protein was purified by dialysis against double-distilled water to obtain precipitated FluIgM-1 which was then dissolved in phosphate-buffered saline (PBS). Affinity chromatography was then carried out, using $FLU_{20}GEL$-coupled Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.). The eluted protein was judged to be pure in that the only bands that were visualized on sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie Blue corresponded to the heavy and light chains of IgM.

Normal rabbit serum used in the precipitation experiments was absorbed by three passages over a column of Sepharose 4B coupled with normal mouse Ig, followed by two passages over a column coupled with HPC76 (IgM) myeloma protein to remove any anti-mouse Ig activity.

Another rabbit antiserum (R647) with strong anti-μ chain activity, raised against purified, polyspecific IgM, was obtained from J. E. Layton (Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Victoria, Australia).

Biosynthetic Labeling Protocol. Hybridoma cells, at a density of 2.5×10^6/ml, were treated with blockading antigen for 1 h at 37°C in DME-10% FCS. Controls were incubated in parallel in the absence of antigen. The cells were then diluted with 10 ml of ice-cold DME and washed by centrifuging at 600 *g* for 10 min through an FCS underlayer. This process was then repeated. (It was found that further washing made no difference to the results.) The pellet was then resuspended in 1 ml of the biosynthetic labeling medium. This consisted of DME containing only 10^{-5} M leucine, 10% FCS, and 20 μCi/ml of L-[6-^{3}H]leucine (Amersham, Eastbourne, England). Incubation was performed in Falcon (No. 2058; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) test tubes for 4 h. Comparison with incubation in 2-ml Petri dishes showed little difference in results, but recovery of cells was facilitated by the use of the test tubes.

After incubation, the cell suspension was centrifuged at 600 *g* for 10 min through an FCS underlayer. Supernates were removed and placed in test tubes. In most instances, $FLU_{20}GEL$ was the antigen employed; 10 μg/ml of collagenase (A grade; Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) was added where it was desirable to digest any $FLU_{20}GEL$ that was present. The nonionic detergent Nonidet P-40 (NP40) (Shell Chemical Co., New York) was then added to a final concentration of 0.5% vol/vol, to the supernates, which were stored at −20°C. The cell pellet was lysed in 0.5% NP40 in buffer 150 mM/liter NaCl, 5 mM/liter EDTA, 50 mM/liter Tris, and 0.2% (wt:vol) sodium azide buffer (NET buffer) plus 10 μg/ml collagenase if residual $FLU_{20}GEL$ was to be digested, and then centrifuged at 2,000 *g* for 10 min to remove debris. The supernates were removed and stored at −20°C.

Plaque-forming Cell (PFC) Assay. FLU-specific IgM antibody secretion was detected using the Cunningham and Szenberg (13) modification of the Jerne hemolytic plaque assay with sheep erythrocytes (SRBC) coated with FLU-conjugated $F(ab')_2$ fragments of rabbit anti-SRBC (FLU-SRBC). The hybridoma cells (50 μl) were mixed with 50 μl of 2% vol/vol FLU-SRBC and a 1:20 dilution of guinea pig serum as a source of complement. The mixture was pipetted into Cunningham chambers which were sealed with paraffin. After 1 h at 37°C the chambers were examined with a colony microscope at magnification of 25, and the number and size of plaques were determined.

Trichloroacetic Acid (TCA) Precipitation. The samples (10 μl) were mixed with 50 μl of 20% FCS in serology tubes, and protein precipitated with 1 ml of ice-cold 20% TCA, which was vortexed and then diluted with 4 ml of 5% TCA. The tubes were then centrifuged at 2,000 *g* for 10 minutes, and the supernate removed. The pellet was dissolved in a small volume of 0.4 N NaOH, reprecipitated as above, centrifuged, and the pellet dissolved in 100 μl of 0.4 N NaOH. This material was then placed in scintillation vials, solubilized with 300 μl of Soluene-350 (Packard Instrument Co., Inc., Downers Grove, Ill.), and 4 ml of scintillation fluid added. Samples were counted on a Packard Scintillation Counter (Packard Instrument Co., Inc.).

Direct Coprecipitation. Direct coprecipitation was carried out by adding 25 μl of pooled FluIgM-1 ascites to 25 μl of the sample, diluting the mix in 200 μl of 0.5% NP40-PBS, vortexing, then adding a previously optimized amount of FLU-BSA to yield a maximal precipitate. The precipitates were incubated at 37°C for 1 h, then at 4°C overnight. Precipitates were washed three times in 0.5% NP40-PBS, then dissolved in 100 μl of 0.1 M acetic acid and prepared for counting as for TCA precipitates.

Indirect Coprecipitation Using Staphylococci. The method of Kessler (14), was used to measure IgM. Briefly, 50 μl of sample, 200 μl of 0.5% NP40-NET buffer and 10 μl of specific anti-μ antiserum were incubated for 1 h at 37°C. After this time, 200 μl of a 10% suspension of heat-killed, formalin-treated Cowan I strain *Staphylococcus aureus* (in 0.5% NP40-NET buffer) was added and incubation continued for a further 15 min. The preparations were washed three times in 0.5% NP40-NET. The final pellet then mixed with 100 μl of 0.1 M acetic acid to elute bound IgM and centrifuged at 2,000 *g* for 10 min. Supernates were removed and counted as before.

The Measurement of Biosynthetically Labeled Antibody by a Solid-Phase Immunoabsorbent. Polyvinyl chloride (PVC) 96-well microtiter plates (Dynatech Corp., Cooke Engineering Co., Alexandria,

Virginia) were treated with FLU-BSA (10^{-4} M fluorescein) for 24 h at room temperature. Any remaining binding sites on the plastic were then blocked with 10% agammaglobulinemic horse serum. Plates were dried and stored at 4°C until they were used.

An aliquot, (50 µl) of each sample of the biosynthetically labeled supernates or cell lysates, was added to a designated well and the plate was incubated at 4°C overnight. The plates were then washed 10 times, cut up with scissors and individual wells transferred to scintillation vials, to which was added 5 ml of 25% Triton X-100 in scintillation fluid and which were counted as before.

Solid-phase Radioimmunoassay. The method of Teale et al. (15) was employed. Samples (50 µl) were added to wells of $FLU_{10}BSA$-coated PVC plates as above. After overnight incubation, the plates were washed twice, and aliquots of ^{125}I-labeled antiserum provided by Dr. J. Teale (The Walter and Eliza Hall Institute of Medical Research), either directed against Fab fragments or against class-specific determinants, were added. The plates were incubated for 24 h, washed 10 times and cut up, individual wells being placed in tubes for counting on a Beckman Autogamma counter (Beckman Instruments, Inc., Electronic Instruments Div., Schiller Park, Ill.).

Thymidine Uptake. 1 µCi [^{3}H]thymidine (Amersham) was added to 1 ml of cell suspension. The cells were harvested on glass fiber filters and washed extensively with distilled water. Radioactivity was measured by transferring the dried filters to scintillation vials, solubilizing with 300 µl of Soluene-350 and adding 4.5 ml of scintillation fluid.

Fluorescence Microscopy. Cells were directly labeled with rhodamine-conjugated sheep anti-mouse Ig (prepared by J. E. Layton), or with the blockading fluorescein conjugates (e.g., $FLU_{20}GEL$). The cells were incubated for various times at 4°C or 37°C before preparation for examination by either fluorescence microscopy or the fluorescence-activated cell sorter (FACS) (Becton, Dickinson FACS Systems, Mountain View, Calif.) (16, 17). In other experiments, cells were incubated with the rabbit anti-FluIgM-1 antiserum for varying times, washed, then stained with rhodamine-conjugated sheep anti-rabbit antiserum for 10 min at 4°C. The cells were then washed and studied by fluorescence microscopy.

In some experiments the cells were washed twice and examined immediately, whereas in other experiments, washed cells were fixed in paraformaldehyde and stored at 4°C. No difference was detected in the fluorescence associated with cells treated by these two methods.

Fc Receptor Rosettes. Washed SRBC were incubated at 37°C with rabbit anti-SRBC antiserum (obtained from B. L. Pike) for 30 min, then washed, and reconstituted to a 5% suspension in normal saline. The rosetting assay was carried out by mixing equal volumes of the SRBC suspension with hybridoma cells or peritoneal exudate cells. The mixtures were incubated at 37°C for 40 min, then examined for rosettes in a hemocytometer.

In experiments investigating the presence of Fc receptors capable of binding IgM, sheep erythrocytes were coated with IgM antibody using serum obtained from mice 4 d after immunizing with sheep erythrocytes, when the bulk of anti-SRBC antibodies are of the IgM class.

Statistical Calculations. The Student's t test was used to determine significance. In general, tests were performed three or four times and means and standard errors are presented.

Results

Anti-Fluorescein-Antibody-secreting Hybridoma. Tissue cultures were examined for growth 14 d after the fusion of NS-1 myeloma cells with spleen cells from mice injected 3 d previously with FLU-POL. Proliferating cells were observed in 25 wells of the 96-well tray and in all wells of the 24-well trays. Supernates from these wells were tested for the presence of anti-FLU antibodies using the radioimmunoassay based on FLU-BSA-coated plates. Antibody was detected in 9 of the 25 small wells, and in most wells of the 24-well trays. Supernates were also tested for the presence of various Ig classes using isotype-specific radioiodinated antisera (Fig. 1). This demonstrated that in all but one well, the secreted immunoglobulin was IgM; in well 19, IgG2a was detected.

Aliquots of cells from each well were frozen in liquid nitrogen. The eight wells

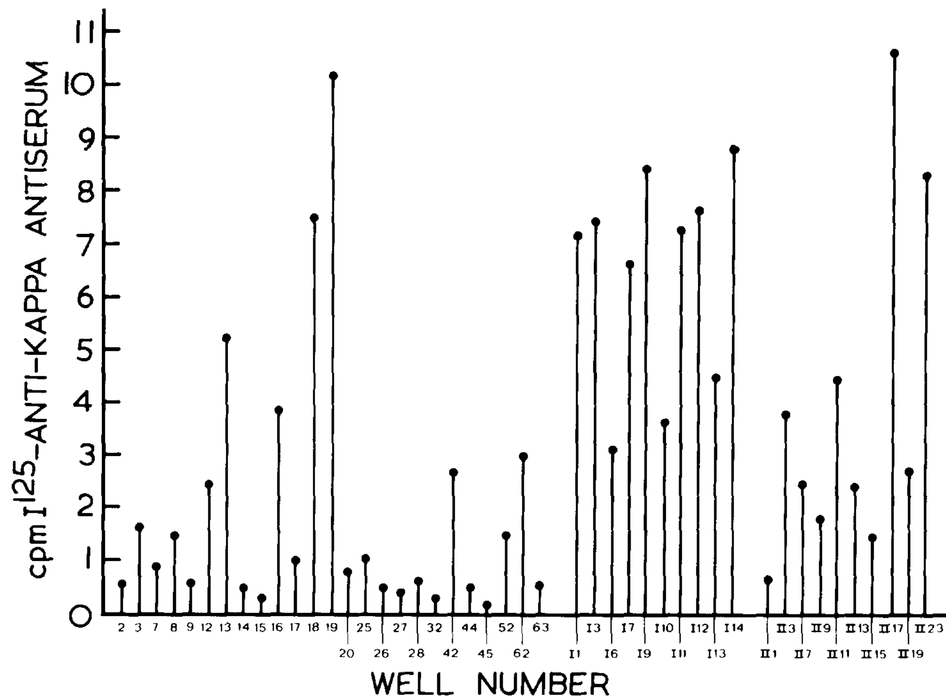

FIG. 1. Measurement of antibody production in wells 2 wk after a hybridoma fusion. The counts per minute of ^{125}I-labeled anti-kappa antiserum used in the RIA are plotted for each well that showed growth 2 wk after fusion of spleen cells from mice immunized with FLU-POL with the NS-1 myeloma line.

showing highest levels of anti-FLU antibody production were cloned by limiting dilution two times (Fig. 1, wells 3, 18, 19, 62, I-1, I-9, II-11, and II-17). Immunoglobulin production in the clones was assayed by RIA and by the hemolytic plaque assay. The clones producing the most Ig produced direct plaques, the diameter and clarity of which correlated with the amount of IgM detected by the RIA. The clones derived from well 19 (IgG2a) and the clones producing lower amounts of IgM antibody (from wells II-11, 3, and I-1) gave hemolytic plaques only in the presence of an enhancing serum. The four clones secreting the highest amounts of IgM have been kept in continuous passage and have been stable for >9 mo, in terms of growth characteristics and immunoglobulin-secretion rates.

The experiments reported in this paper were performed on the anti-FLU secreting clone which was derived from well II-17 and has been designated FluIgM-1. This clone consistently produced high levels of anti-FLU activity, when tested by RIA or the biosynthetic-labeling technique described below. FluIgM-1 also produced the largest direct anti-FLU plaques. These were smaller than antibody-forming cell plaques derived from normal spleen cells, but showed greater homogeneity in size.

The Presence of Surface Ig on FluIgM-1. To investigate whether FluIgM-1 expressed surface immunoglobulin, FluIgM-1 and the parental NS-1 cells (which produce no immunoglobulin) were treated with rhodamine-labeled anti-Ig. The binding was studied by fluorescence microscopy and with the FACS. Significant binding of rhodamine-labeled anti-Ig to FluIgM-1 compared with NS-1 cells was observed using

both techniques. FACS fluorescence profiles demonstrating the difference between the binding rhodamine-labeled, anti-mouse-Ig-treated FluIgM-1 and NS-1 cells are shown in Fig. 2A. Similar experiments showed that the amount of rhodamine-labeled Ig bound to FluIgM-1 was considerably less than that binding to normal B cells (data not shown). Surface immunoglobulin was also demonstrated by incubating FluIgM-1 and NS-1 cells with the anti-FluIgM-1 antiserum, then staining with rhodamine-conjugated sheep anti-rabbit antibody, the fluorescence of the FluIgM-1 cells being strongly positive, whereas NS-1 cells were negative (Fig. 2B).

We also investigated the ability of FluIgM-1 to specifically bind the hapten FLU. FluIgM-1 cells were incubated at 37°C with FLU-conjugated proteins and after washing were analyzed by fluorescence microscopy or by the FACS. NS-1 was again used as a control cell. FluIgM-1 bound FLU_{20}-GEL to a significantly greater extent than did NS-1. In other experiments, it was shown that this binding was not inhibited by unconjugated GEL. Thus, in that FluIgM-1 secreted readily detectable amounts of IgM anti-FLU antibody, bound fluorescein conjugates, and had Ig on the cell surface, it appeared to be a suitable cell line for the study of the antigen-mediated blockade of antibody secretion.

Antigen-mediated Blockade of Hemolytic Plaque Formation. Initial experiments using the hemolytic plaque assay, demonstrated that FluIgM-1 was susceptible to effector-cell blockade. FluIgM-1 cells were incubated with 10–50 μg/ml of FLU_{20}GEL for 1 h at 37°C. FLU_{20}GEL-pretreated cells and control cells were then washed twice and assayed in a FLU-specific hemolytic plaque assay. It can be seen that the pretreatment of the hybridoma cells with FLU_{20}GEL resulted in a reduction in plaque numbers of ~30%, with a similar decrease in diameter (Table I). At lower doses, where plaque numbers were not greatly reduced, plaque size was nevertheless consistently reduced.

Control experiments showed that preincubation of FluIgM-1 cells with either

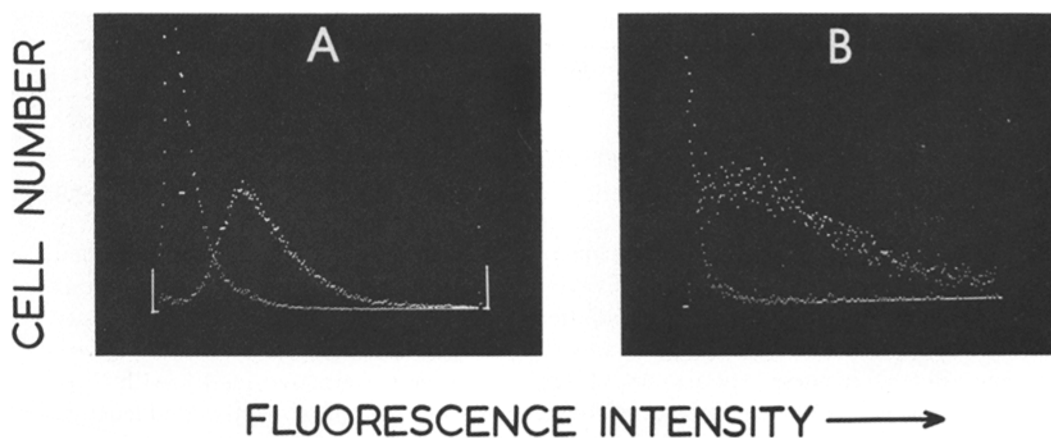

FIG. 2. The presence of surface immunoglobulin on FluIgM-1, detected by anti-immunoglobulin antisera. (A) FluIgM-1 cells and NS-1 cells were labeled with rhodamine-conjugated sheep anti-mouse Ig antiserum. The photograph is a FACS analysis of fluorescence on the vertical axis against cell number on the horizontal axis. The left hand peak is the profile generated for NS-1, the right hand peak is that for FluIgM-1. (B) NS-1 and FluIgM-1 cells were labeled with rabbit anti-IgM antibody, washed, and then rhodamine-conjugated sheep anti-rabbit Ig antiserum was added. The right hand curve is the FluIgM-1 fluorescence profile. The left hand curve is the NS-1 cells fluorescence profile.

TABLE I

The Effect of Preincubation with $FLU_{20}GEL$ on Hemolytic Plaque Formation by FluIgM-1

Pretreatment*	Anti-FLU PFC‡
	100 ± 7§
$FLU_{20}GEL$ 10 μg/ml	82 ± 4
$FLU_{20}GEL$ 25 μg/ml	81 ± 5
$FLU_{20}GEL$ 50 μg/ml	74 ± 3§
$NIP_{33}GEL$ 50 μg/ml	93 ± 8

* Cells were incubated for 1 h at 37°C with the indicated amounts of $FLU_{20}GEL$ or $NIP_{33}GEL$ and then washed twice and resuspended in Hepes-buffered Eagle's medium-FCS.

‡ Plaque assays were performed as described above, counted on a colony microscope, and normalized.

§ $P < 0.02$.

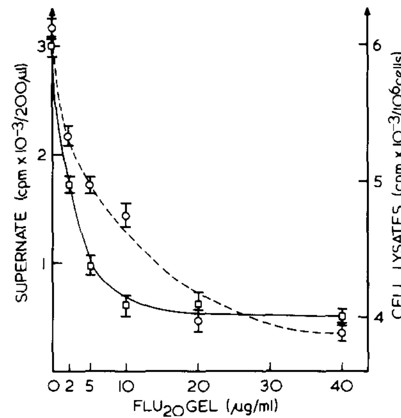

FIG. 3. The suppression of secretion and synthesis of IgM in FluIgM-1, after pretreatment with $FLU_{20}GEL$. Cells were incubated for 1 h at 37°C with varying amounts of $FLU_{20}GEL$, then washed and transferred to medium that contained tritiated leucine. After 4 h of incubation they were analyzed in $FLU_{10}BSA$-coated microtiter trays. Incorporation of tritiated leucine is plotted against amount of antigen use to pretreat the cells for supernates (□) and cell lysates (○).

unconjugated GEL, or GEL conjugated with the unrelated hapten mononitroiodophenyl (NIP), had no such inhibitory effect. These experiments thus demonstrated that the hybrodoma FluIgM-1 was susceptible to effector-cell blockade.

The Effect of FLU Conjugates on Ig Synthesis. The chief advantage of using a cloned cell line as a model for the study of ECB is that direct measurements of biosynthesis can be made on a homogeneous population of cells. A first approach was to quantitate the changes in Ig secretion in ECB and to investigate whether the synthesis of Ig was also affected. In these experiments, FluIgM-1 cells were pretreated for 1 h with FLU conjugate, washed twice, then transferred to medium that contained tritiated leucine. The cells were allowed to incorporate tritiated leucine for 4 h, after which, cell lysates and supernates were assayed for radiolabeled antibody.

Fig. 3 shows the results of an experiment in which FluIgM-1 cells were pretreated with varying amounts of $FLU_{20}GEL$. It can be seen that the amount of newly secreted antibody was substantially reduced after treatment with amounts of FLU_{20}-GEL >2 μg/ml. Furthermore, the content of newly synthesized Ig in cell lysates was also reduced in $FLU_{20}GEL$-pretreated cells. Taken together, these results clearly indicate

that pretreatment of the hybridoma with $FLU_{20}GEL$ markedly reduced total Ig synthesis.

The possibility that the observed reduction in Ig synthesis was an artifact, resulting from the persistence of $FLU_{20}GEL$ or its digestion products, was excluded in three ways. First, the results obtained using $FLU_{10}BSA$-coated microtiter tray system were checked by mixing varying ratios of control supernates with supernates from cells pretreated with $FLU_{20}GEL$. The supernates had been treated with collagenase in the normal way. The results of such an experiment are shown in Table II. It can be seen that results are consistent with those predicted by the assumption of simple mixing of the control and treated supernates. Thus there was no evidence that the treated supernates contained material capable of interfering with binding of anti-FLU antibody to the $FLU_{10}BSA$ plates.

In a second approach, assays of biosynthetically labeled supernates were performed in trays coated with anti-μ-chain antiserum. As shown in Table III, the results of assays using anti-μ-chain-antibody-coated trays were in good agreement with the results obtained with antigen-coated trays (although the sensitivity and background counts of the two systems differed). Finally, a different approach was to measure

TABLE II

The Effect on the Anti-FLU Antibody Assay of Mixing Supernates from Control and Pretreated Cultures

	$[^3H]$Leucine
	cpm/10⁶ cells
Control	15,235 ± 1,094
Pretreated*	7,357 ± 821
Pretreated (25%) + control (75%)‡	12,931 ± 40
Pretreated (50%) + control (59%)	10,532 ± 215
Pretreated (75%) + control (25%)	8,264 ± 72

* Cells were pretreated with 25 μg/ml of $FLU_{20}GEL$ for 1 h at 37°C.
‡ Mixtures of supernates from cultures pretreated with $FLU_{20}GEL$ and control cultures were prepared in the proportions shown, and aliquots assayed with control and pretreated supernates.

TABLE III

Comparison of Assays of FluIgM-1 Antibody Using PVC Plates Coated with Anti-µ-Chain Antibody or FLU_{10} BSA

Experiment number	Pretreatment	Counts per minute of $[^3H]$leucine-labeled FluIgM-1 bound to trays coated with	
		Anti-μ-chain antibody	FLU_{10} BSA
1	—	2,504 ± 76	5,400 ± 161
	$FLU_{20}GEL$ 5 μg/ml	1,685 ± 45*	2,624 ± 33‡
	$FLU_{20}GEL$ 10 μg/ml	1,482 ± 39*	2,138 ± 57‡
2	—	5,963 ± 219	22,147 ± 874
	$FLU_{20}GEL$ 5 μg/ml	4,834 ± 177*	11,157 ± 644‡
	$FLU_{20}GEL$ 10 μg/ml	4,499 ± 135*	8,803 ± 471‡

Cells were pretreated with $FLU_{20}GEL$ for 1 h at 37°C as shown above, then washed twice, and biosynthetically labeled for 4 h. Aliquots were assayed in microtiter plates labeled with either anti-μ-chain antiserum, or with $FLU_{10}BSA$. Two experiments are shown above.
* $0.001 < P < 0.01$ compared to controls.
‡ $P < 0.001$ compared to controls.

biosynthetically labeled antibody by using anti-μ-chain antiserum and protein A-bearing staphylocci to precipitate IgM (Table V).

The close parallels between the results obtained with these methods again indicates that any residual $FLU_{20}GEL$ or the digestion products of the collagenase treatment were not interfering with binding to $FLU_{20}BSA$-coated plates.

Specificity of Blockade of Ig Synthesis Using Other Haptens. In these experiments, $NIP_{33}GEL$ and $DNP_{33}GEL$ were used to pretreat hybridoma cells and the results compared with identically prepared control and $FLU_{20}GEL$-treated cells. The results (Tables IV and V) show that only the $FLU_{20}GEL$ produced blockade and thus that the effect was hapten specific and not related to the carrier protein.

The Effect of Carrier and Conjugation Ratio on Blockade. These experiments investigated the effects of varying the conjugation ratio in conjugates of FLU with BSA, human gamma globulin (HGG), and GEL (Table V). As specific digestion of carrier protein was not possible for BSA and HGG, it was necessary to measure antibody levels with a method that was not interfered with by persisting antigen. Therefore anti-FLU antibody levels were determined by indirect coprecipitation using an anti-μ-chain antiserum and staphylococci.

The results obtained show that blockade was much more readily induced by the highly substituted conjugates. A comparison of 50 μg/ml of FLU_5HGG with 10 μg/ml of $FLU_{24}HGG$ illustrates this point. The total molarity of FLU is nearly identical in both groups but clearly the highly substituted conjugate is far more suppressive. Furthermore, it was evident that HGG conjugates were more suppressive than BSA or GEL conjugates. The larger size of HGG and the flexible nature of this molecule might facilitate interaction with multiple Ig receptors. Taken with the requirement for multivalency, these data suggest that multipoint binding to surface receptors may be an important step in ECB of Ig synthesis.

Effect of Pretreatment with FLU_{20}-GEL on Total Cell Protein Synthesis. To investigate whether the observed reduction in Ig synthesis in blockaded cells was specific, or reflected rather a general suppression of protein synthesis; total TCA precipitable counts were compared with those present in immunoprecipitates or in the FLU_{10}-BSA-coated wells.

In Fig. 4 the results of two experiments are tabulated as a percentage of control counts. The TCA-precipitable counts in the supernates were reduced in $FLU_{20}GEL$-treated cultures. This is understandable as Ig forms a major component of proteins released into the supernate by this cell line (A. W. Boyd. Unpublished data.).

TABLE IV

The Effect of Specificity of Antigen on Blockade of Ig Secretion

Pretreatment	[^{3}H]Leucine
	cpm/10^6 cells
—	12,673 ± 516
$FLU_{20}GEL$ 50 μg/ml	3,350 ± 125*
$NIP_{33}GEL$ 50 μg/ml	13,052 ± 285
$DNP_{33}GEL$ 50 μg/ml	11,676 ± 460

FluIgM-1 cells were incubated with the antigen for 1 h at 37°C, washed twice, then biosynthetically labeled. Supernates were assayed in $FLU_{10}BSA$-coated microtiter trays.

* $P < 0.001$ for control versus $FLU_{20}GEL$ treated; control versus $NIP_{33}GEL$ or $DNP_{33}GEL$ treated: $P > 0.5$.

TABLE V

The Effect of Multivalency and the Nature of the Carrier on the Induction of ECB in FluIgM-1 Cells

	Pretreatment	Supernate	Cell lysate
FluIgM-1	—	4,688 ± 97	2,099 ± 87
	$NIP_{33}GEL$ (10 μg/ml)	4,373 ± 66	2,045 ± 74
	$NIP_{33}GEL$ (50 μg/ml)	4,852 ± 43	2,328 ± 81
	$FLU_{3.7}GEL$ (10 μg/ml)	4,510 ± 144	1,980 ± 31
	$FLU_{3.7}GEL$ (50 μg/ml)	4,104 ± 97	2,228 ± 81
	$FLU_{20}GEL$ (10 μg/ml)	934 ± 44*	1,211 ± 56*
	$FLU_{20}GEL$ (50 μg/ml)	832 ± 13*	1,046 ± 74*
	$FLU_{3}BSA$ (10 μg/ml)	3,879 ± 200*	2,121 ± 96
	$FLU_{3}BSA$ (50 μg/ml)	2,528 ± 58*	2,498 ± 28
	$FLU_{11}BSA$ (10 μg/ml)	1,209 ± 73*	1,251 ± 40*
	$FLU_{11}BSA$ (50 μg/ml)	795 ± 56*	939 ± 29*
	$FLU_{5}HGG$ (10 μg/ml)	4,435 ± 155	2,073 ± 87
	$FLU_{5}HGG$ (50 μg/ml)	2,998 ± 150*	1,631 ± 71‡
	$FLU_{11}HGG$ (10 μg/ml)	832 ± 10*	2,047 ± 38
	$FLU_{11}HGG$ (50 μg/ml)	671 ± 18*	1,857 ± 17‡
	$FLU_{24}HGG$ (10 μg/ml)	934 ± 44*	1,211 ± 56*
	$FLU_{24}HGG$ (50 μg/ml)	832 ± 13*	1,046 ± 74*
NS-1		253 ± 30	703 ± 29

Cells were blockaded with FLU conjugates as shown, with the substitutions indicated by subscript in each case. The supernates and cell lysates were analyzed by staphylococcal immunoprecipitation using anti-μ-chain antiserum.

* $P < 0.001$ versus control.

‡ $P < 0.01$, other results not significantly different from controls.

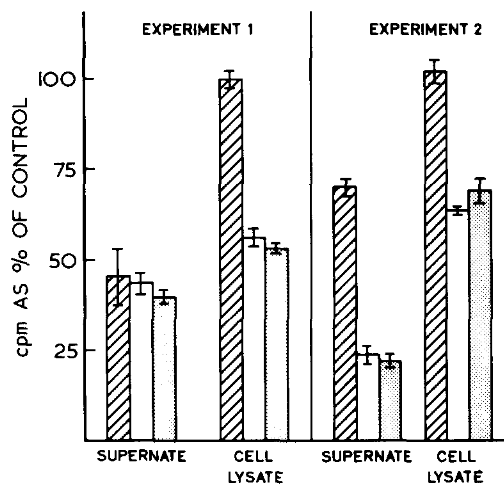

FIG. 4. Comparison of immunoglobulin specific and total protein [^{3}H]leucine incorporation in control and $FLU_{20}GEL$-pretreated FluIgM-1 cells. Two experiments are shown that compare incorporation of tritiated leucine into supernates and cell lysates by three methods: (▨) TCA precipitation, (□) immunoprecipitation (direct coprecipitation with $FLU_{10}BSA$), (▩) $FLU_{10}BSA$-coated microtiter trays.

However, the cell lysates show no change in TCA-precipitable counts relative to controls, despite a significant reduction in Ig-specific counts. Thus blockade seems to affect Ig synthesis specifically.

DNA Synthesis Rate. The incorporation of tritiated thymidine by the hybridoma was measured at various times after pretreatment with $FLU_{20}GEL$ and compared with that in both control cells and cells continuously exposed to $FLU_{20}GEL$. At each time point, thymidine was added and incorporation over the next 4 h was measured. As is seen in Table VI, no significant differences were observed between these three groups. Further, the cell numbers in all three groups at 24 and 48 h were never >10% different, with no significant trend in either direction detectable over a number of experiments. Thus the mechanism of blockade does not appear to involve either accelerated cell death or a change in numbers of dividing cells.

Recovery from Blockade. The duration of the blockade of Ig synthesis that followed pretreatment of the hybridoma cells with antigen was investigated by pretreating cells with 10 μg/ml of $FLU_{20}GEL$ for 1 h, washing them and resuspending them for varying periods in DME-FCS at 37°C in 2-ml Petri dishes. After varying periods, $FLU_{20}GEL$-pretreated cells were compared with control cells. At each time point, aliquots of cells were washed and resuspended in [^{3}H]leucine-containing medium. Biosynthetic labeling was carried out for 4 h, after which, amounts of ^{3}H-labeled antibody were determined as before. The results are plotted in Fig. 5, and indicate that suppression is maximal between 0–6 h, but only slight at 24 h, and by 48 h, pretreated cells have recovered and synthesize Ig at a rate comparable to that of control cells. The continuous presence of antigen in the culture medium was shown to inhibit this recovery (Fig. 5).

Fc Rosette Assay on FluIgM-1 Cells. One possibility not hitherto excluded by published data, is that the mechanism of effector-cell blockade may involve the Fc receptor. For example Fc receptors could bind secreted antibody that had complexed with antigen bound to surface immunoglobulin receptors. To investigate whether the hybridoma had Fc receptors, rosetting assays were performed.

In the IgG rosetting assay, peritoneal exudate cells showed numerous rosettes (>40%) but FluIgM-1 cells were entirely negative. To investigate the possibility that a FluIgM-1 may have an Fc receptor specific for μ chain, rosetting was also carried out with erythrocytes coated with mouse IgM anti-SRBC antibodies. Once again, no rosettes were observed (nor in this case were they present in assays of peritoneal

TABLE VI

The Effect of ECB on DNA Synthesis in FluIgM-1 Cells

Treatment of cells	[^{3}H]Thymidine
	cpm/2 × 10^5 cells
	4,207 ± 397
Pretreatment for 1 h with $FLU_{20}GEL$*	4,154 ± 331
Continuous presence of $FLU_{20}GEL$ (10 μg/ml)‡	4,475 ± 422

FluIgM-1 cultures were pretreated with 10 μg/ml of $FLU_{20}GEL$ for 1 h, then washed twice. Incorporation was carried out for 4 h.

* Cells were incubated with [^{3}H]thymidine in medium containing no antigen.

‡ 10 μg/ml of $FLU_{20}GEL$ was added to the medium with the [^{3}H]thymidine.

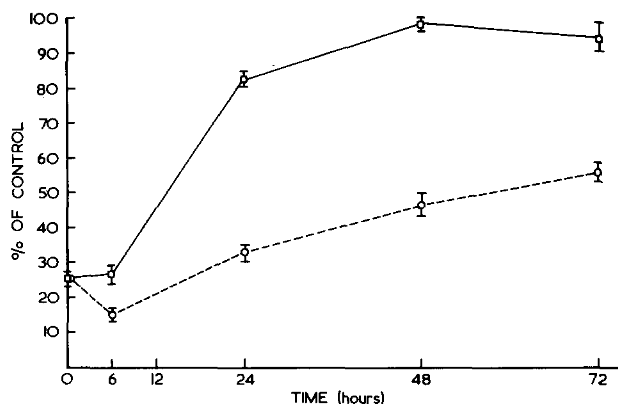

FIG. 5. Secretion rate ([^{3}H]leucine incorporation in 4 h) is plotted against time for cells exposed to 10 μgm/ml of $FLU_{20}GEL$ for 1 h. In one group (□), cells were washed twice and allowed to recover (1-h antigen pretreatment). In the second group (○), antigen was present continuously.

exudate cells performed in parallel). Hence, we may conclude that FluIgM-1 does not have Fc receptors and thus that Fc receptor binding is not a necessary step in ECB.

Fluorescence Microscopy of Cells During Antigen Pretreatment. FluIgM-1 cells were exposed to $FLU_{20}GEL$ at 37°C, and aliquots removed after various times and prepared for fluorescence microscopy. The cells showed a characteristic sequence of changes (Table VII). By 30 min, fluorescence microscopy showed that most of the fluorescence was localized in randomly distributed bright spots. With longer times, these spots increased in size and decreased in number, but the general pattern was preserved and capping was never seen. The pattern correlated well with that observed in cells treated with rhodamine-conjugated sheep anti-mouse Ig antiserum, or with other FLU conjugates.

Fluorescence Microscopy and FACS Analysis of $FLU_{20}GEL$-pretreated Cells. It was important to determine whether the duration of blockade coincided with the persistence of antigen in association with the cells. Therefore cells were treated with the blockading antigen $FLU_{20}GEL$ at 37°C for 1 h, washed twice and transferred to fresh medium. After varying periods, cells were washed and fixed with paraformaldehyde and stored at 4°C until analysis was carried out.

Cells fixed immediately after the pretreatment periods showed the characteristic pattern of bright fluorescent spots described above. After ~4 h these spots gradually reduced in number and intensity, and had virtually disappeared by 24 h.

Table VIII shows a FACS analysis of pretreated cells, and demonstrates that there is a steady decline in the number of fluorescent cells. This result correlated well, both with fluorescent microscopy observations and also with the recovery from blockade measured by biosynthetic labeling. Thus the blockade of Ig synthesis in FluIgM-1 appears to correlate with the association of antigen (FLU) with the cell.

In the light of previous observations that suggested that extracellular antigen was critical for the maintenance of ECB, it was of interest to determine how much of the $FLU_{20}GEL$ associated with blockaded FluIgM-1 cells was extracellular. To examine this question, cells were treated with collagenase at various times after pretreatment with FLU-GEL. The effect of treatment with collagenase on fluorescence was then

TABLE VII

Distribution of Fluorescence on FluIgM-1 during the 1-h $FLU_{20}GEL$ Pretreatment, and during the Subsequent 23 h When No Free Antigen Was Present

Time	Microscopic appearance of cell-associated antigen
0*	Only faint, diffuse surface fluorescence.
5 min* (a)	Again, the diffuse "ring" staining is evident, but bright points of fluorescence are beginning to develop.
10 min*	Many brightly fluorescent spots, randomly distributed.
30 min*	Bright spots are larger, and appear spherical in shape; there are >30/cell. Cytoplasmic staining is noted.
1 h‡	The size of the bright spots continues to increase, but the number is reduced to 20–30/cell.
2 h‡	There is a continuing reduction in the number of fluorescent spots(10–20/cell).
6 h‡	Brightness reduced further as a result of continuing decrease in fluorescent spots.
24 h‡	The cells are much fainter and a proportion of cells have no visible fluorescence, the pattern is still that of a few (usually less than five) fluorescent spots. At this stage, brightly fluorescent vesicles are seen floating free in the medium.

The fluorescent microscopic appearance is recorded for hybridoma cells at various times after the addition of $FLU_{20}GEL$ (10 μg/ml) to the cultures. At 1 h, cells were washed twice and then cultured in medium that contained no antigen. At each time point cells were centrifuged through FCS and fixed in paraformaldehyde, and then prepared for microscopy.

* Observations during $FLU_{20}GEL$ pretreatment.

‡ Follow-up observations of blockaded cells.

TABLE VIII

FACS Analysis of the Fluorescence of Blockaded FluIgM-1 Cells

Time*	Fluorescent cells‡
h	%
0	43.4
1	56.3
2	46.9
6	32.1
24	6.6

* FACS analyses of the fluorescence intensity, performed at each time point, of aliquots of cells that had been treated with $FLU_{20}GEL$ for 1 h at 37°C and then washed twice.

‡ The fluorescent cells (>channel 20 on the FACS) are expressed as a percentage of a 4×10^4 cell sample for each time point.

determined by fluorescence microscopy. At all time points at which fluorescence remained detectable on the cells, collagenase treatment resulted in a significant reduction of FLU staining. In particular, the bright spots were removed. The residual fluorescence was mainly diffuse, although in some cells there remained condensations of fluorescence which appeared to be perinuclear. This persistence of extracellular

antigen during the period when blockade was observed is consistent with previous data on the role of extracellular antigen in mediating blockade.

Discussion

The experiments reported in this paper describe a hybridoma cell line, FluIgM-1, which secretes an IgM antibody specific for the hapten FLU. When these cells were exposed to highly multivalent FLU conjugates, the secretion rate of antibody was suppressed as indicated by hemolytic plaque assays (Table I) and biosynthetic labeling (Fig. 3). The rapid onset and dose-response of the phenomenon as seen in FluIgM-1, parallelled closely the ECB phenomenon in normal spleen antibody-forming cells (AFC) (1-4).

As in normal AFC, the hybridoma cells could only be blockaded by molecules bearing multiple, specific antigenic determinants (Tables IV and V). The data also suggest that the nature of the carrier may have been important, as HGG conjugates were more suppressive than BSA conjugates of similar hapten substitutions. This may reflect the relatively greater likelihood of multipoint binding by the larger, more flexible HGG conjugates compared with BSA conjugates. Taken together with the observation that FLU conjugates capable of inducing blockade form surface aggregates (Table VII), this suggests that cross-linking of Ig receptors may be an important factor in inducing and maintaining blockade.

Several observations were pertinent to the role of antigen aggregates in the ECB phenomenon. Parallel measurement of Ig secretion rate, with direct observation of the cell-associated fluorescent aggregates, showed that the clearance of antigen from the cells correlated with recovery of Ig secretion rates. Furthermore, at all times when cell-associated antigen remained detectable, at least a portion was accessible to enzymatic digestion. This was in accord with previous observation on normal spleen AFC (2), indicating that surface bound antigen was necessary to induce and maintain ECB.

One possibility not hitherto excluded was that Fc receptors, which appear to be involved in other examples of B cell down regulation (18), may be involved in the mechanism of ECB. The demonstration that ECB can be induced by nonimmunoglobulin antigens (e.g., $FLU_{20}GEL$, $FLU_{11}BSA$) does not exclude a role for the Fc receptor. Secreted antibody could bind to any type of multivalent antigen bound to the cell surface, forming a local antibody-antigen complex, and the Fc pieces of the secreted antibody in this complex could bind to Fc receptor on the cell. This study excludes a critical role for the Fc receptor in ECB however, as FluIgM-1 was shown to lack receptors for IgG or IgM isotypes. Thus Ig receptor may be the sole inducing signal for ECB.

In conclusion, induction of ECB in the hybridoma cell line described, closely parallels that in IgM-secreting splenic AFC. This hybridoma furthermore, offers the unique advantage of allowing direct visualization of the specific antigen (FLU), and as it is a homogeneous cell population, facilitates the investigation of the molecular events resulting in suppression of Ig synthesis. Finally, further study of the mechanism of ECB in this hybridoma may shed light on other phenomena in which Ig-receptor binding is important, such as B cell proliferation and tolerance induction.

Summary

A mouse hybridoma cell line, FluIgM-1, which secretes IgM specific for the hapten fluorescein (FLU) was developed to allow detailed analysis of the effector-cell blockade (ECB) phenomenon, in which contact of antibody-forming cells (AFC) with specific antigen results in marked reduction of antibody secretion.

Treatment of hybridoma cells with highly substituted FLU conjugates (e.g., FLU_{20}gelatin) resulted in inhibition of plaque formation. The data indicated close parallels with the ECB of normal spleen AFC, both in speed of onset and the dose of antigen required. The inhibition of antibody secretion was confirmed with a biosynthetic-labeling procedure which demonstrated that this was a result of reduced Ig synthesis. The inhibitory effect appeared to be confined to antibody synthesis, in that total protein synthesis, DNA synthesis, and cell-doubling times were unaffected.

The association of FLU conjugates with the cells during and following ECB was studied directly using fluorescence microscopy and the fluorescence-activated cell sorter. These experiments showed that FLU conjugates capable of causing blockade aggregated on the cell surface, that the clearance of cell-associated antigen correlated with recovery from ECB, and that at all times when cell associated antigen was detectable, a portion remained bound to the cell surface and was susceptible to enzymatic removal. The latter observations supported previous findings suggesting that ECB was mediated by extracellular antigen. The direct observation of aggregates of antigen on the surface of blockaded cells is consistent with a mechanism involving cross-linking of Ig receptors. Finally, Fc receptors were not present on hybridoma cells, excluding their involvement in induction of ECB.

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