PRODUCTION OF TARGET-SPECIFIC EFFECTOR CELLS USING HETERO–CROSS-LINKED AGGREGATES CONTAINING ANTI-TARGET CELL AND ANTI-Fcγ RECEPTOR ANTIBODIES

By

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A number of types of effector cells express surface receptors that are specific for the Fc portion of IgG (FcγR).[1] When such cells encounter target cells that have been opsonized with IgG antibodies, they form conjugates with the target cells by binding the opsonizing antibody to their FcγR. Subsequently, the effector cells either lyse or phagocytose the targets, depending upon the cell types that form the conjugates (1–4). In this paper, we demonstrate that conjugate formation and lysis can also be induced by using covalently cross-linked antibody heteroaggregates that contain both anti-FcγR antibodies and antibodies directed against a target cell determinant. When effector cells bind such heteroaggregates to their FcγR, they will then specifically bind and lyse target cells that have not been opsonized, but which express the appropriate antigen.

The purpose of these studies is twofold. First, we wished to produce specific effector cells that are more potent lytic agents than the classical antibody-dependent cell-mediated cytolysis (ADCC) effector cell. In vitro studies (5–9) have demonstrated that ADCC is readily inhibited by either immune complexes or high concentrations of monomeric IgG, both of which are present in the physiological environment. In the past, several investigators (10–13) found that ADCC effector cells that have been incubated with IgG antibodies and washed retain small amounts of antibody on their surfaces and can lyse target cells that bear the appropriate antigens. Such "armed" effector cells, however, are only weakly lytic and require high effector-to-target ratios to mediate lysis. More recently (14), we have found that a number of procedures, collectively termed

"franking" (to distinguish them from arming), lead to nearly irreversible binding of relatively large amounts of antibody to effector cells. Franked effector cells specifically and efficiently lyse target cells that have not been pretreated with antibody, but which express the appropriate antigen. Moreover, ADCC mediated by franked effector cells is much less inhibitable by immune complexes than is classical ADCC. Franked effectors have been produced from murine and human cells (14, 15) and have been shown to lyse both red cell and tumor targets. These results have been confirmed (16) and extended (17) in other laboratories. The use of antibody heteroaggregates, described in this paper, provides a simpler and more efficient method of franking effector cells.

The second purpose of this study is to examine the mechanism of ADCC. By using Fab and F(ab')$_2$ fragments in the heteroaggregates, we have established that the normal Fc-FcγR interaction is not required for lysis. On the other hand, if conjugates are formed using heteroaggregates containing anti–major histocompatibility complex (MHC) class I molecules instead of anti-FcγR, very little lysis is observed. These results suggest a role for FcγR in both the conjugate-forming and lytic steps of ADCC.

Materials and Methods

Materials. RPMI 1640 was purchased from Gibco Laboratories (Grand Island, NY), glutamine, penicillin, and streptomycin were from the National Institutes of Health media unit, and phosphate-buffered saline (PBS), pH 7.2, was from Biofluids (Rockville, MD). Fetal calf serum (Microbiological Associates, Walkersville, MD) was heated to 56°C for 1 h before use. Trinitrobenzenesulfonic acid (TNBS) and dimethyl suberimidate (DMS) were obtained from Pierce Chemical Co. (Rockville, IL). Sodium [^{51}Cr]chromate (1 mCi/ml, in isotonic saline) and sodium [^{125}I]iodide (17 Ci/mg in aqueous saline) were purchased from New England Nuclear (Boston, MA). N-Succinimidyl-3-(2-pyridyldithiol) propionate (SPDP) was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Fluorescein isothiocyanate (FITC) and substituted rhodamine isothiocyanate (XRITC) were purchased from Research Organics, Inc. (Cleveland, OH). FACS (fluorescence-activated cell sorter) medium is Hanks' balanced salt solution without phenol red (Biofluids), pH 7.2, containing 2% bovine serum albumin (Miles Laboratories, Inc., Elkhart, IN) and 0.2% sodium azide. The culture medium used in these experiments was RPMI 1640 containing 5–10% fetal calf serum, 0.03% glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Antibody. Rabbit anti-2,4-dinitrophenyl (DNP) antibodies were isolated from immune serum by affinity chromatography followed by gel filtration as described previously (18). The F(ab')$_2$ fragment was prepared by pepsin digestion at pH 4.3 (14) and it gave a single band of M_r 100,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The rat anti-mouse FcγR monoclonal antibody (mAb) 2.4G2 (19) and the mouse anti-human polymorphonuclear leukocyte (PMN) FcγR mAb 3G8 (20) were grown as ascites in nude mice and isolated by gel filtration and ion-exchange chromatography as described (19, 20). The Fab of 2.4G2 was prepared by papain digestion (19) and did not contain any intact antibody as judged by SDS-PAGE. Sepharose-linked 2.4G2 was kindly provided by Dr. Howard Dickler (Immunology Branch, NCI). Cells producing 34-1-2, a mouse IgG2a mAb with specificity for H-2K^d and H-2D^d, were a kind gift from Dr. David Sachs (21). Ascites fluid from 34-1-2, produced in nude mice, was precipitated with 50% saturated ammonium sulfate, redissolved in water, and dialyzed against borate-buffered saline (BBS), pH 8.5. It was then fractionated on an Ultrogel AcA 34 (LKB Instruments, Inc., Rockville, MD) column in BBS and protein eluting at the IgG position was pooled and further purified by protein A–Sepharose affinity chromatography. Bound 34-1-2 was eluted with 2 M KSCN and dialyzed immediately against BBS. The F(ab')$_2$ fragment of 34-1-2 was prepared by digesting the intact protein with 10% (wt/wt) pepsin/mAb for 4

h at 37°C in 0.15 M sodium acetate, pH 4.0. The sample was then neutralized and passed over a protein A–Sepharose column; the nonadherent protein was further purified by gel filtration over AcA 34, and was homogeneous by SDS-PAGE.

Cross-linking of Antibodies. Heteroaggregates of 2.4G2 or 3G8 and rabbit anti-DNP IgG antibodies were prepared using SPDP according to the manufacturer's protocol. The detailed procedure for one preparation was as follows. Rabbit anti-DNP IgG antibodies (2 ml, 12 mg/ml) and 2.4G2 (4.6 ml, 5.2 mg/ml) were each dialyzed against 0.1 M potassium phosphate, 0.1 M NaCl, pH 7.5 (coupling buffer), and incubated separately for 2 h at room temperature with eightfold molar excesses of SPDP (125 μl of a 3.2-mg/ml solution of SPDP in ethanol was added to each sample). The 2.4G2 was redialyzed against coupling buffer and the anti-DNP was dialyzed against 0.1 M sodium acetate, 0.1 M NaCl, pH 4.5. Dithiothreitol was then added to the anti-DNP to a final concentration of 0.02 M. After 30 min at room temperature, the anti-DNP was passed through a Pharmacia PD10 column equilibrated with coupling buffer, and immediately added to the 2.4G2. After 4 h incubation at room temperature, 1 mg of iodoacetamide was added and the protein was eluted on a 2.6 × 90 cm Ultrogel AcA 22 column in BBS plus 0.02% sodium azide. Polymerized material was collected in two fractions and concentrated using a Millipore CX-10 immersible membrane. Total yields from the column were 16 mg of the heavy cross-linked fraction, 13 mg of the lighter cross-linked fraction, and 6 mg of monomeric IgG. Heteroaggregates of 2.4G2 (or 2.4G2 Fab) and rabbit $F(ab')_2$ anti-DNP were prepared similarly, except that the SPDP 2.4G2 was reduced at pH 4.5 and then incubated with unreduced anti-DNP at pH 7.5.

Anti-DNP $F(ab')_2$ was cross-linked with 34-1-2 $F(ab')_2$ using DMS in order to avoid reduction of the $F(ab')_2$ fragment into the monovalent $F(ab')$ fragments. The two proteins were dialyzed against 0.2 M Tris, pH 8.5, and 4.3 mg of each was mixed and concentrated to 0.2 ml with a CX-10 immersible membrane. An eightfold molar excess of DMS over total protein was added (20 ml of an 11.2-mg/ml DMS solution in water), and the protein was incubated for 3 h at room temperature. The protein was then fractionated on AcA 22, yielding 0.9 mg of the heavy cross-linked fraction, 1.8 mg of the lower molecular weight cross-linked fraction, and 2.1 mg of non–cross-linked $F(ab')_2$.

Binding Studies. Antibodies were radiolabeled with ^{125}I by a modified chloramine T method (22). The amount of radiolabeled antibody associated with cells was determined as described (23).

Target Cells. Chicken red blood cells (CRBC) were labeled with ^{51}Cr as described (14). Tumor cell targets, EL4 (a C57BL mouse lymphoma line) and RDM4 (an AKR mouse leukemia line) were grown in tissue culture and radiolabeled by incubating 0.25 ml of cells (~2 × 10^7 cells/ml) with 0.25 ml ^{51}Cr for 60 min at 37°C in a 5% CO_2 incubator and washing twice. P815 (a DBA/2 mouse mastocytoma line) was grown in tissue culture and radiolabeled with ^{125}I-UdR (Amersham Corp., Arlington Heights, IL) as described (24). For TNP labeling, CRBC were incubated with 10 mM TNBS and tumor cell targets with 5 mM TNBS in PBS, pH 7.2, for 10 min at 37°C, and washed twice. Control cells were incubated with PBS only. TNP CRBC used as targets in classical ADCC experiments were incubated with subagglutinating dilutions on anti-DNP antibodies for 30 min at 37°C. After extensive washing, target cells were counted and resuspended to the desired concentration in culture medium.

Effector Cells. Cells from the $P388D_1$ mouse macrophage line were grown in spinner culture (25), harvested, washed twice, and resuspended to a final concentration of 2–4 × 10^7 cells/ml in culture medium containing the desired concentration (usually 10–20 μg/ml) of cross-linked antibody (franking) or monomeric anti-DNP IgG (arming). Control cells were incubated in culture medium only. After incubation at 0°C for 30–45 min, cells were washed twice and resuspended to the desired concentration in culture medium. Resident peritoneal exudate cells (PEC) were taken from 6–8-wk-old C57BL/6 mice (Frederick Cancer Research Center, Frederick, MD) of either sex, as described (26). To obtain activated cells, ~10^7 viable bacilli Calmette-Guérin (BCG) (TMC 1011 lot 10; Trudeau Institute, Saranac Lake, NY) were injected intraperitoneally per mouse. PEC were then harvested 10–35 d after injection, as described (26). Human PMN were isolated

from heparinized blood from normal donors by the method of Bass et al. (27). Contaminating erythrocytes were lysed with distilled water, and the cells were washed twice in Hanks' balanced salt solution. These cells were ~95% PMN by differential leukocyte counts. Both mouse PEC and human PMN were franked with cross-linked antibody as for the P388D$_1$ cells, except that 3G8 × anti-DNP heteroaggregates were used for franking the PMN.

Inhibition Studies.   Immune complexes used in inhibition experiments were prepared by mixing equal (wt/wt) amounts of the mouse myeloma protein UPC 120 (28) and affinity-purified rabbit anti-mouse IgG2b antibody (kindly supplied by Dr. Jeffrey Bluestone, Immunology Branch, NCI). Effector cells were incubated with immune complexes for 30 min at 0°C before addition of target cells. In hapten inhibition studies, graded concentrations of DNP ϵ-aminocaproic acid were added to the target cells before they were mixed with the effectors. When immune complexes or hapten were used as inhibitors, they remained in the medium during the cytotoxic phase of the experiment. In other studies, 2.4G2 was incubated with effector cells for 30 min at 0°C before adding cross-linked antibody. The cells were washed before being used in the assay.

Chromium Release Assay.   Cytotoxicity was measured as previously described (14). Briefly, various numbers of effector cells were added to 10^5 CRBC target cells or 2×10^4 tumor target cells in the wells of U-bottomed microtiter plates. In classical ADCC experiments involving tumor targets, anti-DNP antibodies (10 μg/ml final concentration) were also added to the wells. The total volume in all wells was 200 μl. After 20 h incubation for CBRC or 4 h incubation for tumor target cells at 37°C in 5% CO_2 and 100% humidity, supernatants were harvested by using the Titertek wick harvesting system (Flow Laboratories, Inc., Rockville, MD) and were assayed for the amount of ^{51}Cr released. Maximum lysis was determined by incubating target cells with an equal volume of 2 M HCl. Spontaneous lysis was measured in the supernatants of cultures containing 10^5 ^{51}Cr-labeled CRBC and 10^6 unlabeled CRBC in assays involving CRBC targets, or 2×10^4 ^{51}Cr-labeled tumor targets in medium alone in assays for tumor target cytolysis. The percent lysis was determined from the formula: percent lysis = 100 × [net cpm (experimental release − spontaneous release)/net cpm (maximum release − spontaneous release)].

Assay for Dual Specificity of Heteroaggregated Antibody.   The anti-DNP activity of 2.4G2 × anti-DNP was assayed by hemagglutination of TNP-coated sheep red blood cells (SRBC). SRBC were incubated in 10 mM TNBS for 10 min at 37°C and washed twice. TNP SRBC (50 μl of a 0.5% suspension) were then added to 75 μl of serial dilutions of the cross-linked antibodies in microtiter plates. The plates were incubated 20 h at 4°C and read visually for hemagglutination. To show that anti-DNP activity was linked with anti-FcγR activity, heteroaggregates (0.75 ml, 10 μg/ml) were repeatedly adsorbed on P388D$_1$ cells (4 × 10^7 cells/adsorbtion) before the hemagglutination assay. To correct for nonspecific adsorbtion and dilution effects, a control adsorption containing P388D$_1$ cells and a saturating concentration of free 2.4G2 was done in parallel with the test adsorbtion.

Conjugate Formation Assay.   The method of Segal and Stephany (29) was used. Briefly, P388D$_1$ cells were washed once with PBS and resuspended at ~10^7 cells/ml in 10 ml PBS. The solution was made 10 μM in FITC by adding 100 μl of a 1-mM solution of FITC in ethanol. After 10 min incubation at 37°C, the cells were centrifuged and washed twice in culture medium. CRBC were washed once in PBS and resuspended at ~10^7 cells/ml in 10 ml PBS containing 10 mM TNBS. Next, 100 μl of 0.5 mM XRITC in ethanol was added and the cells were incubated for 10 min at 37°C, pelleted, and washed twice with culture medium. Some cells were then incubated with subagglutinating dilutions of anti-DNP antibodies for 30 min at 37°C and washed twice with culture medium. Both P388D$_1$ cells and CRBC were washed in FACS medium and resuspended at the desired cell density (usually at 2 × 10^7 cells/ml). Equal volumes of FITC P388D$_1$ cells and XRITC CRBC were then mixed and maintained in suspension by rotation (5 rotation/min) in a 4°C cold room for 2 h. Aliquots were removed, diluted to ~10^6 cells/ml, and analyzed for conjugate formation using a FACS II dual-laser cell sorter (B-D FACS Systems, Becton, Dickinson, & Co., Sunnyvale, CA). The cytometer sequentially analyzes each particle for red and

green emission; free CRBC appear red only, free P388D$_1$ cells green only, and conjugates both red and green. The percentages of particles detected as conjugates, free P388D$_1$ cells, or free CRBC were determined by integrating the appropriate peaks in dual-parameter contour plots.

Results

Binding of Anti-FcγR × Anti-DNP F(ab')$_2$ to Effector and Target Cells. Rabbit anti-DNP F(ab')$_2$ antibodies were chemically cross-linked to 2.4G2, a monoclonal rat anti-mouse FcγR antibody, and the cross-linked material [termed anti-FcγR × anti-DNP F(ab')$_2$] was isolated by gel filtration. To demonstrate that the binding capacities were not destroyed by the cross-linking procedure, we labeled the anti-FcγR × anti-DNP F(ab')$_2$ with ^{125}I and measured its binding to P388D$_1$ cells (Fig. 1A) and to TNP CRBC (Fig. 1B). Fig. 1 shows that anti-FcγR × anti-DNP F(ab')$_2$ binds saturably to P388D$_1$ cells, and that binding is inhibitable by unlabeled anti-FcγR mAb but not by DNP hapten. The anti-FcγR × anti-DNP F(ab')$_2$ also binds to TNP CRBC, and binding is inhibitable by DNP hapten but not by unlabeled anti-FcγR mAb. The binding curve in Fig. 1B does not reach a plateau in the concentration range tested, which is characteristic of the way in which our heterogeneous rabbit anti-DNP antibody preparations bind to TNP-treated target cells (30). Fig. 1 demonstrates that anti-FcγR × anti-DNP F(ab')$_2$ binds to FcγR on P388D$_1$ cells and to TNP groups on TNP CRBC. To establish what proportion of the cross-linked molecules retained both functional activities, we adsorbed anti-FcγR × anti-DNP heteroaggregates on P388D$_1$ cells. We then tested the material after adsorbtion for the presence of anti-DNP activity by its ability to agglutinate TNP SRBC. The results showed that ~70% of anti-DNP activity was adsorbed after three consecutive incubations with P388D$_1$ cells. Since anti-DNP activity was still being removed after the last adsorbtion, it is likely that >70% of the molecules possessing anti-DNP activity also bound to FcγR.
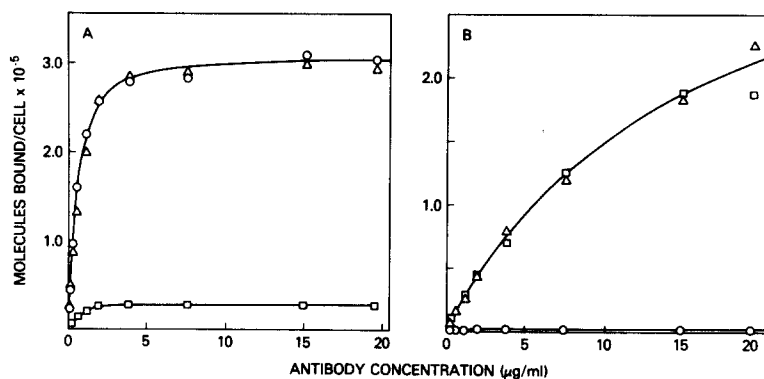

FIGURE 1. Binding of anti-FcγR × anti-DNP F(ab')$_2$ antibody to P388D$_1$ cells and to TNP CRBC. The number of molecules (based on a molecular weight of 1.5×10^5) of ^{125}I-labeled cross-linked antibody bound to the cells (ordinate) is plotted against the molar concentration of free antibody in which the cells were incubated (abscissa). Binding of radiolabeled anti-FcγR × anti-DNP F(ab')$_2$ to P388D$_1$ cells (A) and to TNP CRBC (B). (△) Cross-linked antibody alone. (□) Cross-linked antibody in the presence of a 40-fold excess of unlabeled anti-FcγR mAb. (○) Cross-linked antibody in the presence of 10^{-4} M DNP ϵ-aminocaproate.

Lysis of TNP CRBC by Franked P388D₁ Cells. P388D₁ cells were incubated with either anti-FcγR × anti-DNP or with anti-FcγR × anti-DNP F(ab')₂, washed, and tested for their ability to lyse CRBC or TNP CRBC. P388D₁ cells treated with either preparation of hetero–cross-linked antibodies lysed TNP CRBC but not untreated CRBC, and maximal lysis was achieved when P388D₁ cells were treated with a 5–10-μg/ml solution of cross-linked material (Fig. 2). Although 10 mM TNBS was used in most experiments to modify CRBC, lysis could also be detected on target cells treated with as little as 0.5 mM TNBS (data not shown). Specificity of lysis was further demonstrated by showing that DNP hapten inhibited lysis in a dose-dependent manner (Table I). To conform with previous publications (14, 15), we shall refer to the attachment of antibody to
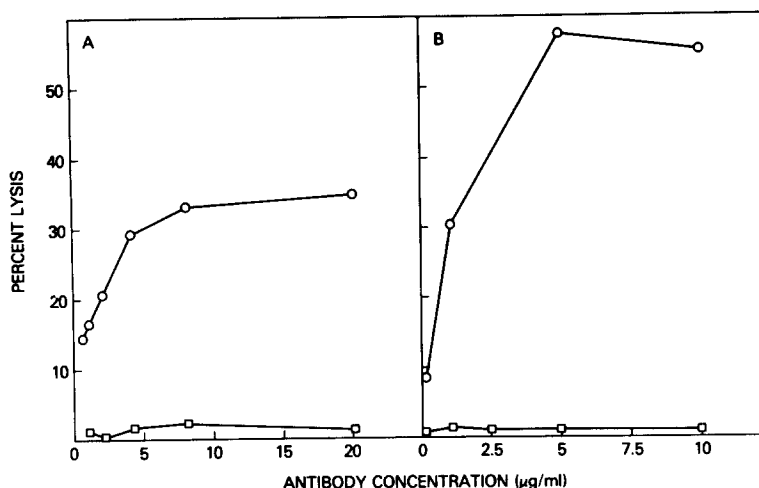

FIGURE 2. Lysis of TNP CRBC (○) and CRBC (□) by P388D₁ franked with anti-FcγR × anti-DNP (*A*) and with anti-FcγR × anti-DNP F(ab')₂ (*B*) at a 10:1 effector-to-target ratio.

TABLE I

DNP Hapten Inhibition of TNP CRBC Lysis by Franked P388D₁ Cells

| | Percent inhibition of lysis | |
| | P388D₁ franked with: | |
Concentration of DNP hapten	Anti-FcγR × anti-DNP*	Anti-FcγR × anti-DNP F(ab')₂‡
M		
10^{-10}	<0§	5
10^{-8}	<0§	24
10^{-6}	50	61
10^{-4}	94	95

* P388D₁ cells, incubated with 10 μg/ml anti-FcγR × anti-DNP, were tested for lysis of TNP CRBC in a 20-h ^{51}Cr release assay, at an effector-to-target ratio of 10:1. Lysis in the absence of inhibitor was 34.5%.

‡ Same as above except P388D₁ cells were incubated with 10 μg/ml anti-FcγR × anti-DNP F(ab')₂. Lysis in the absence of inhibitor was 50.6%.

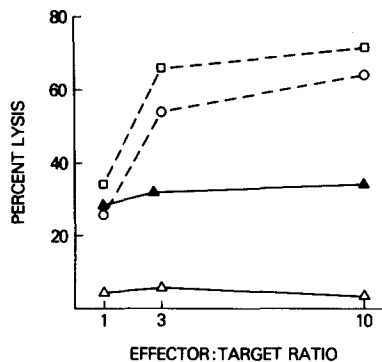§ Lysis was greater in the presence of inhibitor than in its absence.

FIGURE 3. Lack of requirement of the Fc portion of IgG for cytolysis. Lysis of TNP CRBC by P388D$_1$ cells in a 20-h ^{51}Cr release assay. (□) P388D$_1$ cells franked with 25 μg/ml anti-FcγR × anti-DNP tested against TNP CRBC. (○) P388D$_1$ cells franked with 25 μg/ml anti-FcγR Fab × anti-DNP F(ab')$_2$ tested against TNP CRBC. (▲) Untreated P388D$_1$ cells tested against TNP CRBC coated with anti-DNP antibody. (△) Untreated P388D$_1$ cells tested against TNP CRBC.

FcγR on effector cells as "franking." The data show that franked P388D$_1$ cells will specifically lyse TNP CRBC, and that the Fc portions of the anti-DNP antibodies are not required for lysis. These data do not, however, rule out the possibility that the Fc piece of the anti-FcγR mAb is involved in lysis. This was tested by cross-linking the Fab fragment of the anti-FcγR mAb with the F(ab')$_2$ of anti-DNP. Fig. 3 shows that P388D$_1$ cells franked with this material lysed TNP CRBC almost as effectively as P388D$_1$ cells franked with heteroaggregates of the intact antibodies. Therefore, lysis mediated by cells franked with antibody heteroaggregates does not require the Fc portions to be present on either antibody.

To test for lysis of "innocent bystander" cells, franked P388D$_1$ cells were incubated with equal numbers of ^{51}Cr-labeled, unmodified CRBC, and unlabeled TNP CRBC. In this experiment (data not shown), specific lysis of the unmodified target cells was not observed, showing that lysis of innocent bystander cells does not occur in this system.

The length of time that P388D$_1$ cells remain cytolytic after franking is shown in Table II. In this experiment, P388D$_1$ cells were franked by the usual procedure at 0°C, and at time zero the cells were warmed to 37°C and periodically tested for lysis against TNP CRBC. Untreated control P388D$_1$ cells were tested for lysis against antibody-coated target cells. The results of Table II show that the lytic activity of franked cells drops rapidly in the first 5 h of incubation, and still further after an additional 11 h. By contrast, very little ADCC activity is lost by untreated control cells. Because P388D$_1$ cells rapidly endocytose bound immune complexes (31), it is remarkable that detectable cytolytic activity remains for as long as 16 h after franking.

In Table III, the results from five different experiments are summarized, comparing the lysis of antibody-coated targets (classical ADCC) with lysis of haptenated targets by armed or franked effector cells. These data show that, in this system, the franked P388D$_1$ cells are much more potent effectors than the armed cells, and that lysis mediated by the franked effectors is significantly

TABLE II

Percent Lysis after Incubation of Effectors in Medium*

Time	Percent specific lysis	
	ADCC with franked effectors[‡]	Classical ADCC[§]
h		
0	66.8	36.0
5	24.8	38.2
16	5.7	25.9

* At an effector-to-target ratio of 10:1 in a 20-h ^{51}Cr release assay.

[‡] P388D$_1$ cells were franked once with 50 µg/ml anti-FcγR × anti-DNP at time zero and incubated in suspension at 37°C. At various times, the cells were washed twice and an aliquot was tested for cytotoxicity against TNP CRBC.

[§] Same as above except that the effector cells were treated with medium only and were tested against TNP CRBC in the presence of 5 µg/ml of anti-DNP antibody.

TABLE III

Lysis of Target Cells by P388D$_1$ Cells*

Treatment of effector cells	Experiment	Percent lysis[‡] of:		
		Antibody-coated TNP CRBC	TNP CRBC	CRBC
Incubated with medium only	1	12.2 (0.5)	−0.1 (4.1)	−0.5 (0.4)
	2	34.5 (1.0)	2.5 (0.5)	0.4 (0.4)
	3	13.8 (1.1)	3.0 (0.9)	2.9 (0.6)
	4	11.1 (1.4)	2.4 (0.1)	−0.6 (0.7)
	5	31.4 (5.7)	3.5 (0.6)	1.2 (0.3)
Incubated with[§] anti-DNP antibody	1	16.8 (0.9)	7.5 (4.2)	−1.0 (0.4)
and washed (armed)	2	40.3 (1.4)	12.6 (0.8)	1.0 (0.4)
	3	24.8 (2.8)	11.9 (0.9)	0.4 (0.4)
	4	13.5 (1.7)	5.9 (0.7)	−1.5 (0.5)
	5	16.9 (2.0)	5.7 (0.8)	1.5 (0.4)
Franked with[§] anti-FcγR × anti-DNP	1	50.4 (2.4)	44.5 (5.7)	−0.8 (0.4)
	2	46.3 (3.8)	34.8 (2.3)	1.5 (0.5)
	3	107.5 (2.8)	90.8 (1.6)	−0.1 (0.5)
	4	40.5 (5.5)	53.3 (2.8)	−0.9 (0.5)
	5	62.7 (3.6)	49.5 (2.1)	0.9 (0.2)

* At effector-to-target ratios of 10:1.

[‡] Means of triplicate samples followed by standard errors in parenthesis.

[§] P388D$_1$ cells were treated with 10–20 µg/ml of anti-DNP antibody or anti-FcγR × anti-DNP.

greater than that observed when untreated P388D$_1$ cells lyse antibody-coated targets.

Formation of Conjugates Between TNP CRBC and Franked P388D$_1$. Conjugates between franked P388D$_1$ cells and TNP CRBC were detected using a flow cytometric technique, in which P388D$_1$ cells and TNP CRBC were labeled with green- and red-emitting fluorophores, respectively. Cells were mixed at 4°C to

allow conjugates to form but to prevent subsequent lysis, and the suspensions were then analyzed using a dual-laser flow cytometer; free P388D$_1$ cells appear green only, free CRBC red only, and conjugates appear as single particles that are both red and green (e.g., Fig. 4D). Fig. 4 demonstrates that when untreated P388D$_1$ cells were incubated with TNP CRBC, no conjugates formed (panel A). By contrast, when untreated P388D$_1$ cells were incubated with antibody-coated TNP CRBC (panel B), or when franked P388D$_1$ cells were incubated with TNP CRBC (panel D), stable conjugates were readily detected. Armed P388D$_1$ cells (panel C) formed many fewer conjugates than did the franked P388D$_1$ cells. In other experiments (data not shown), we found that DNP hapten inhibited the formation of conjugates between franked P388D$_1$ cells and TNP CRBC, and that pretreatment of P388D$_1$ cells with anti-FcγR mAb prevented P388D$_1$ cells from forming conjugates with antibody-coated TNP CRBC.

Inhibition of Lysis and Conjugate Formation by Immune Complexes. Classical ADCC—that is, lysis of antibody-coated target cells by FcγR-bearing effector cells is readily inhibited by immune complexes (5–9). However, because of the extremely high affinity with which 2.4G2 binds to FcγR, we would expect lysis mediated by franked effectors to be much less sensitive to immune complex inhibition. We therefore tested the ability of immune complexes to inhibit cytolysis and conjugate formation using franked, armed, or untreated effector cells. In Fig. 5A, graded concentrations of immune complexes were incubated with effectors and targets in a 20-h ^{51}Cr release assay. This figure shows that both the lysis of antibody-coated target cells by untreated P388D$_1$ cells and the
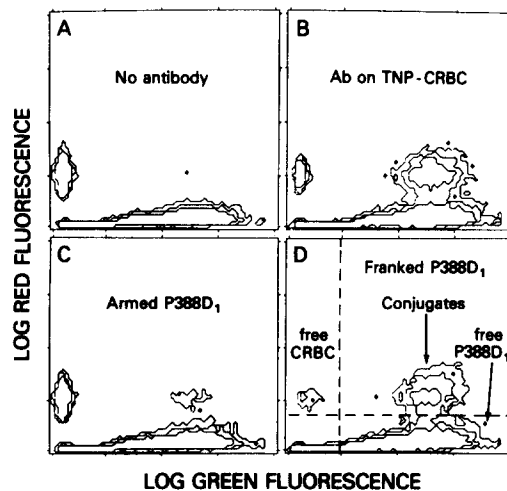

FIGURE 4. Extent of conjugate formation between P388D$_1$ cells and CRBC. FITC-labeled P388D$_1$ cells were mixed with XRITC-labeled CRBC on a rotator in a 4°C cold room for 2 h and then samples were taken for cytometric analysis. Contours were drawn through points containing 10, 20, and 40 particles. A total of 5 × 10^4 particles was analyzed in each sample. (A) Untreated P388D$_1$ cells and TNP CRBC. 1.3% of the particles fall in the conjugate range. (B) Untreated P388D$_1$ cells and TNP CRBC coated with anti-DNP antibody. Percent conjugates, 18.8. (C) Armed P388D$_1$ cells (incubated with anti-DNP antibody and washed) and TNP CRBC. Percent conjugates, 3.2. (D) Franked P388D$_1$ cells (anti-FcγR × anti-DNP) and TNP CRBC. Percent conjugates, 15.5.
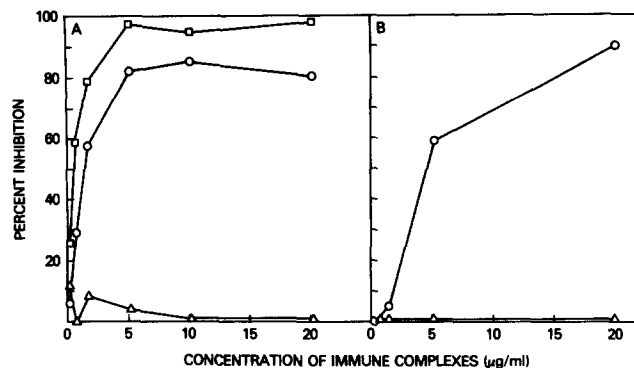
FIGURE 5. Inhibition of lysis and conjugate formation by immune complexes. Immune complexes (mouse IgG2b plus rabbit anti-mouse IgG2b) were first added to effector cells, followed by addition of the target cells. In both assays, the immune complexes were present during the entire course of the assay. (A) Inhibition of lysis in a 20-h ^{51}Cr release assay at a 10:1 effector-to-target ratio. (B) Inhibition of conjugate formation as measured by flow cytometry (see Fig. 4). (O) Untreated P388D₁ cells interacting with TNP CRBC coated with anti-DNP antibody. In the absence of inhibitor, the percent lysis was 13.8 and the percent conjugates 24.5. (□) Armed P388D₁ cells interacting with TNP CRBC. In the absence of inhibitor, the percent lysis was 11.9. Inhibition of conjugate formation could not be tested because too few conjugates (3.2%) formed in the absence of inhibitor. (△) Franked P388D₁ cells (anti-FcγR × anti-DNP) interacting with TNP CRBC. In the absence of inhibitor, the percent lysis was 90.8 and the percent conjugates was 15.5.

lysis of TNP CRBC by P388D₁ cells armed with anti-DNP antibodies are readily blocked by low concentrations of immune complexes. By contrast, lysis of TNP CRBC by P388D₁ cells franked with anti-FcγR × anti-DNP is totally resistant to inhibition. As expected, the inhibition of conjugate formation followed the same pattern (Fig. 5B); in this experiment, armed effector cells could not be tested for inhibition because too few conjugates formed in the absence of immune complexes.

Linkage of Target Cells to FcγR Is Necessary for Lysis. To test whether linkage to FcγR was required for lysis mediated by franked effector cells, we formed conjugates in which the target cells were bound to MHC class I molecules on the effector cells rather than to FcγR. This was done by cross-linking F(ab')₂ fragments from 34-1-2, a monoclonal antibody with specificity for K^d and D^d molecules, to F(ab')₂ fragments from rabbit anti-DNP antibodies. P388D₁ cells (which are of the H-2^d haplotype) were then incubated with this material [anti-K^{d}D^d F(ab')₂ × anti-DNP F(ab')₂], and their abilities to form conjugates and lyse TNP CRBC were compared with those of P388D₁ cells franked with anti-FcγR × anti-DNP. P388D₁ cells treated with anti-K^{d}D^d F(ab')₂ anti-DNP F(ab')₂ were even more effective at forming conjugates than were the P388D₁ cells franked with anti-FcγR × anti-DNP (Fig. 6A). By contrast, only the cells franked with anti-FcγR × anti-DNP mediated a high degree of lysis (Fig. 6B). Since a different cross-linking reagent (DMS) was used to form the anti-K^{d}D^d F(ab')₂ × anti-DNP F(ab')₂ aggregates than was used to make anti-FcγR × anti-DNP (SPDP; see Materials and Methods), it was possible that the difference in lytic activities could have arisen from the difference in cross-linking reagents. However, we found
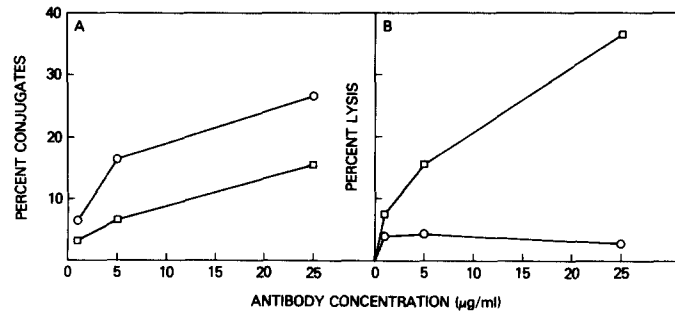
FIGURE 6. The requirement of linkage to FcγR for lysis of TNP CRBC by P388D₁ cells. P388D₁ cells were treated with various concentrations of anti-FcγR × anti-DNP (□) or anti-KᵈDᵈ F(ab')₂ × anti-DNP F(ab')₂ (○) and analyzed for conjugate formation with TNP CRBC (A) or tested for cytotoxicity against TNP CRBC in a 20-h ⁵¹Cr release assay (B) at a 10:1 effector-to-target ratio. In the absence of antibody, the percent conjugates was 1 and the percent lysis was 0.7.

TABLE IV

Inhibition of Conjugate Formation and Lysis

| | P388D₁ treated with:* | |
Inhibitor*	Anti-KᵈDᵈ F(ab')₂ × anti-DNP F(ab')₂	Anti-FcγR × anti-DNP
	Percent conjugates with TNP CRBC	
None	26.8	15.5
Anti-FcγR mAb	23.6	0.6
Anti-KᵈDᵈ mAb	6.2	14.6
	Percent lysis of TNP CRBC‡	
None	3.1 (1.1)	36.7 (0.9)
Anti-FcγR mAb	0.7 (0.9)	0.5 (0.5)
Anti-KᵈDᵈ mAb	3.8 (0.3)	29.9 (0.6)

* P388D₁ cells were pretreated with 0.5 mg/ml antibody inhibitor or with medium for 30 min at 0°C. Cross-linked heteroaggregates (25 µg/ml) were then added and the cells were incubated for an additional 30-min period at 0°C and washed.
‡ Means of triplicate samples followed by standard errors in parenthesis. At effector-to-target ratios of 10:1.

that when the anti-FcγR × anti-DNP aggregates were formed using DMS instead of SPDP, they were still active (data not shown).

The data in Table IV show the results of experiments in which P388D₁ cells were preincubated with a 20-fold excesses of either anti-FcγR mAb or anti-KᵈDᵈ mAb before treatment with antibody heteroaggregates. As expected, preincubation with free anti-FcγR mAb prevented conjugate formation and lysis mediated by P388D₁ cells treated with anti-FcγR × anti-DNP, and preincubation with free anti-KᵈDᵈ mAb inhibited conjugate formation by P388D₁ cells treated with anti-KᵈDᵈ F(ab')₂ × anti-DNP F(ab')₂. When cells franked with anti-FcγR × anti-DNP were pretreated with anti-KᵈDᵈ, only minor effects upon conjugate formation and lysis were observed, which demonstrates that the anti-class I antibody

TABLE V

Lysis of Target Cells by Franked Mouse and Human Effector Cells

				Percent lysis	
Experiment	Effector cells	Target cells	Effector-to-target ratio	Franked effector cells	Untreated* effector cells
1	Mouse resident PEC‡	TNP CRBC	10	36.0	10.2
2	Mouse BCG-activated PEC§	TNP EL4	20	19.2	7.4
3	Mouse BCG-activated PECǀ	TNP P815	15	5.5	−1.2
4	Human PMN¶	TNP CRBC	10	83.7	5.6
5	Human PMN**	TNP RDM4	75	12.8	−1.1

* Incubated with medium only.
‡ PEC from untreated mice franked with 50 μg/ml anti-FcγR (2.4G2) × anti-DNP in a 20-h ^{51}Cr release assay.
§ PEC from BCG-treated mice franked with 50 μg/ml anti-FcγR (2.4G2) × anti-DNP in a 6-h ^{51}Cr release assay.
ǀ Same as above except that cytolysis was measured in a 6-h ^{125}I-UdR release assay.
¶ PMN franked with 100 μg/ml anti-FcγR (3G8) × anti-DNP in a 20-h ^{51}Cr release assay.
** PMN franked with 1 mg/ml anti-FcγR (3G8) × anti-DNP in a 3.5-h ^{51}Cr release assay.

did not suppress lysis. In an attempt to trigger lysis in nonlytic conjugates, $P388D_1$ cells were first treated with anti-FcγR, then with anti-K^dD^d F(ab')$_2$ × anti-DNP F(ab')$_2$, and finally with TNP CRBC. Instead of triggering lysis, pretreatment of the effector cells with anti-FcγR inhibited the small amount of lysis (3.1%) mediated by the anti-K^dD^d F(ab')$_2$ × anti-DNP F(ab')$_2$-treated effectors. This suggests that FcγR were involved in lysis mediated by even these effectors. Such an involvement could arise, for example, if the anti-K^dD^d F(ab')$_2$ × anti-DNP F(ab')$_2$ preparation contained a small amount of contaminating intact antibody. Further attempts to trigger lysis in nonlytic conjugates by adding immune complexes or Sepharose-linked 2.4G2 to preformed conjugates also failed (data not shown). Therefore, the cross-linking of FcγR in conjugates where the target cells are linked to class I molecules instead of directly to FcγR is not a lytic signal.

Lysis of CRBC and Tumor Targets by Franked Primary Effector Cells. The data presented in Table V demonstrate that franked primary murine and human effector cells can lyse CRBC and tumor targets. Franked resident PEC will lyse TNP CRBC (experiment 1) and, when activated with BCG, they will lyse tumor targets as well (experiments 2 and 3). However, the extent of lysis of tumor targets is much lower than for CRBC, and, when assayed by ^{51}Cr release (experiments 1 and 2), PEC mediate a relatively high amount of antibody-independent lysis. In another series of experiments, franked human PMN were tested for lysis against CRBC and tumor targets. To frank human PMN, the anti-FcγR antibody 3G8 was cross-linked with anti-DNP antibodies. The 3G8 antibody binds to FcγR on human PMN and a subset of lymphocytes, but not to FcγR on monocytes (20). The avidity of 3G8 for human FcγR is reported to be substantially lower than that of 2.4G2 for the murine FcγR (20). The data of Table V show that franked human PMN will lyse both CRBC and tumor targets, although in order to detect lysis against tumor targets, high concentrations of franking antibody and high effectors-to-target ratios are required.

Discussion

In classical ADCC, antibody-coated target cells are bound to FcγR on effector cells and subsequently lysed. Because we would expect ADCC in vivo to be inhibited by circulating IgG or immune complexes (32), we previously developed procedures by which antibody was artificially attached in vitro to the effector cell with high avidity, in order to override the normal in vivo suppression of ADCC. Such franked effectors were produced by incubating cells with relatively high concentrations (e.g., 1 mg/ml) of antibody in the presence of polyethylene glycol, and then sedimenting them through a mixture of phthalate oils (14). While the chemical basis for franking was not clear, this procedure resulted in relatively large amounts of radiolabeled antibody becoming cell associated, and rendered the cells potent effectors with the same specificity as the antibody franked to them (14, 15). Nevertheless, the earlier franking procedures were cumbersome and used large amounts of antibody, which was not all bound to FcγR on the cell surface, and some of which eluted from the cells during experimentation.

In this paper, we describe a new, more specific method for franking mouse and human effector cells. In the mouse, the franking antibody is a heteroaggregate of the 2.4G2 anti-FcγR mAb covalently cross-linked to anti-DNP antibodies. This reagent binds to FcγR, not by the normally weak interaction between FcγR and Fc portions of IgG antibodies (22), but instead by the extremely high-affinity interaction between the 2.4G2 combining site and FcγR (19). As a result, the antibody with specificity for the target cell (in this paper, anti-DNP) becomes tightly attached to FcγR when low concentrations of heteroaggregates (e.g., 5 μg/ml) are incubated with effector cell populations. Cells franked by this procedure are rendered specifically cytotoxic for the target cell (Fig. 2, Tables I and II). In addition, the cytolysis mediated by these cells, in contrast to classical ADCC, is not inhibited by immune complexes (Fig. 5).

While the mouse often serves as a good experimental model for in vivo and in vitro studies, mouse cells are notoriously poor effectors of ADCC especially against nucleated target cells (2, 3, 33). Human K cells, a subset of peripheral blood lymphocytes, and monocytes, however, are potent mediators of ADCC against most antibody-coated targets, including tumor cells (2, 3, 34–36). Unfortunately, a high-avidity monoclonal antibody against FcγR on human monocytes and K cells is not yet available. The 3G8 antibody binds with relatively low avidity to FcγR on PMN (which are less effective mediators of ADCC against tumor targets than are monocytes and K cells [2]) and cross-reacts with FcγR on a subset of lymphocytes (20). While heteroaggregates containing 3G8 can produce franked effector cells from human PMN, these cells are only weakly lytic against tumor targets (Table V). Moreover, high concentrations of heteroaggregates are required to successfully frank PMN. Nevertheless, the results of Table V demonstrate that franking can produce lytic effectors from several different types of cells from at least two different species. It is likely, therefore, that when high-avidity antibodies against human monocyte and K cell FcγR become available, more potent human effectors against tumor targets will be produced using appropriate heteroaggregates, and that lysis mediated by such effectors will not be inhibited by monomeric IgG or immune complexes.

Several interesting observations emerge from these studies regarding the mechanism of ADCC. First, the data of Fig. 3 show that ADCC can occur when $Fc\gamma R$ are linked to the target cells through interactions different from those that normally occur between the ligand and receptor. However, linkage of targets to effectors through at least two other cell surface components, the K^d and D^d molecules, does not result in lysis (Fig. 6). Therefore, conjugate formation per se is not sufficient for lysis. Rather, the data suggest that ADCC requires, in addition to conjugate formation, other processes involving $Fc\gamma R$. To test whether the binding of antibody or ligand to $Fc\gamma R$ could trigger lysis in nonlytic conjugates, we treated such conjugates with either 2.4G2 (Table IV), soluble immune complexes, or 2.4G2 bound to Sepharose beads. In no case did treatment of $Fc\gamma R$ in nonlytic conjugates lead to lysis, which suggests that $Fc\gamma R$ must be linked directly to the target cell in order for lysis to occur. Since extensive cross-linking of $Fc\gamma R$ in nonlytic conjugates (by immune complexes or 2.4G2 Sepharose) did not trigger lysis, we also conclude that either cross-linking of $Fc\gamma R$ is not a lytic signal or alternatively, that cross-linking of $Fc\gamma R$ does trigger lysis, but that it must occur locally at the region of intercellular contact. The precise nature of the lytic signal remains one of the most intriguing questions concerning the mechanism of ADCC.

Summary

Rabbit anti-2,4-dintrophenyl (DNP) antibodies or their $F(ab')_2$ fragments were chemically cross-linked to the anti-mouse $Fc\gamma R$ monoclonal antibody 2.4G2 or to its Fab fragment. $P388D_1$ cells were incubated with heteroaggregates between 2.4G2 and anti-DNP (anti-$Fc\gamma R$ × anti-DNP) and washed. The resulting cells lysed 2,4,6-trinitrophenyl chicken erythrocytes (TNP CRBC) in a hapten-specific manner. The lysis was inhibited by free hapten but was resistant to inhibition by immune complexes. Other cells coated with antibody heteroaggregates also mediated lysis of TNP-modified target cells. For example, mouse resident peritoneal exudate cells (PEC) lysed TNP CRBC and bacillus Calmette-Guérin-activated PEC lysed both TNP CRBC and TNP tumor targets. Human neutrophils, when incubated with heteroaggregates containing the anti-human neutrophil $Fc\gamma R$ antibody 3G8 and anti-DNP also lysed TNP CRBC and TNP-modified tumor cells. To test whether linkage to $Fc\gamma R$ was required for lysis, $F(ab')_2$ fragments from the anti-K^dD^d monoclonal antibody 34-1-2 were cross-linked to anti-DNP $F(ab')_2$ fragments. $P388D_1$ cells (which express K^d and D^d) were then incubated with these heteroaggregates and washed, and their abilities to form conjugates and lyse TNP CRBC were compared with those of $P388D_1$ cells treated with anti-$Fc\gamma R$ × anti-DNP. In both cases, $P388D_1$ cells formed conjugates. However, only the cells treated with anti-$Fc\gamma R$ × anti-DNP mediated lysis to a significant extent. We conclude that heteroaggregates containing anti-$Fc\gamma R$ and anti-target cell antibodies can be used to create potent effector cells against red cell and tumor targets and that bridging of effectors with target cells directly to $Fc\gamma R$ on effector cells is required for lysis.

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