THE Fab FRAGMENT OF A DIRECTLY ACTIVATING MONOCLONAL ANTIBODY THAT PRECIPITATES A DISULFIDE-LINKED HETERODIMER FROM A HELPER T CELL CLONE BLOCKS ACTIVATION BY EITHER ALLOGENEIC Ia OR ANTIGEN AND SELF-Ia

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The phenomenon of major histocompatibility complex (MHC)[1] restriction has by necessity become the cornerstone of all models of helper and cytotoxic T cell antigen recognition. Alloreactivity, historically the first example of T cell recognition of MHC determinants, has been variously viewed as an inexplicable property of a subset of T cells or as a cross-reaction of self-MHC–restricted cells. The finding that monoclonal sources of T cells can be activated either by antigen and self-MHC or by allogeneic MHC alone at frequencies that mimic mixed cell populations (1) strengthens the view that alloreactivity is indeed a cross-reactive byproduct of the molecular mechanism of antigen and self-recognition. However, it is not known if a single receptor complex can recognize both self-Ia plus foreign antigen and non–self-Ia. Recently (2–7), probes in the form of monoclonal antibodies specific for individual clones of T cells have become available to directly identify the cell surface structures involved in antigen and Ia recognition and to dissect the cell biology of the process of T cell activation.

This study uses a murine helper T cell clone that is induced to proliferate either by hen's egg conalbumin and I-A^k or by I-A^b alone. As previously described (2), these cloned T cells can also be activated by a monoclonal antibody that recognizes a cell surface determinant unique to this clone. We now demonstrate that this antibody can mimic antigen and Ia in the activation of this clone at concentrations as low as 10^{-11} M. In monovalent form, this antibody no longer activates but rather blocks specific activation by I-A^b or by antigen and I-A^k, strongly supporting the hypothesis that a single molecular species is indeed involved in recognition of both moieties. The target antigen of this antibody is a cell surface glycoprotein, a disulfide-linked heterodimer. Both surface and internal forms of this protein have been identified by immunoprecipitation and

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[1] *Abbreviations used in this paper:* FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; IL-1, interleukin 1; LPS, lipopolysaccharide; LSM, lymphocyte separation medium; MHC, major histocompatibility complex; MIg, mouse immunoglobulin; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RaMBr, rabbit anti-mouse brain serum; SDS, sodium dodecyl sulfate; TdR, thymidine.

two-dimensional gel analysis. The ability of a monoclonal antibody directed against this molecule to activate cells at molar concentrations equivalent to those observed in hormone or lymphokine activation and, in monovalent form, to inhibit T cell activation by antigen or allogeneic Ia, almost certainly identifies this molecule as the antigen/Ia, as well as the allogeneic Ia, T cell receptor.

Materials and Methods

Animals

Animals were either purchased from The Jackson Laboratory, Bar Harbor, ME or raised in our colony at Yale University. All animals were between 2 and 6 mo of age at the time they were used.

T Cell Clones

The production and maintenance of cloned helper T cell lines D10.G4.1 and D8 have been described in detail previously (2). All experiments were performed with cells harvested at least 8 d after stimulation with antigen and feeder cells.

Antigens

Conalbumin and ovalbumin were obtained from the Sigma Chemical Co., St. Louis, MO.

Antibodies

3D3, Monoclonal anti-D10. The production of this BALB.K anti-D10 monoclonal antibody has been described previously (2). Antibody was purified from hybridoma culture supernatants by protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) affinity chromatography (8) or from supernatants of hybridoma cultures grown in serum-free medium (9) by ammonium sulfate precipitation. Antibody concentrations were determined spectrophotometrically at a wavelength of 280 nm.

Fab fragment of 3D3. Protein A-Sepharose affinity-purified 3D3 was adjusted to 1 mg/ml in phosphate-buffered saline (PBS) containing 0.1% sodium azide, 4 mM EDTA, and 0.01 M 2-mercaptoethanol. 0.4 U of papain attached to carboxymethyl cellulose beads (Sigma Chemical Co.) were added per milliliter of antibody solution. The reaction tube was flushed with nitrogen and rotated at 37°C for 18 h. The reaction was terminated by the removal of the papain beads by centrifugation and 1 M Tris base was added to the supernatant to bring the pH to 8. The Fab fragment was purified from remaining intact immunoglobulin and the Fc fragment by a second round of protein A-Sepharose affinity chromatography. The flow-through, containing the Fab fragment, was dialyzed against PBS before use. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis followed by silver staining by the method of Merril et al. (10) was used to confirm the presence of an intact Fab fragment and the absence of detectable whole antibody or Fc fragments. Fab fragment concentrations were determined spectrophotometrically at a wavelength of 280 nm.

Rabbit anti-mouse brain serum (RaMBr). This serum was a gift from Dr. B. Jones, Yale University, and has been described previously (11).

Rabbit anti-mouse immunoglobulin (anti-MIg). A hyperimmune serum was prepared as described previously (12) and purified by MIg-Sepharose affinity chromatography.

Interleukin 1 (IL-1)-rich P388D1 Supernatant

This partially purified supernatant was obtained from lipopolysaccharide (LPS)-activated P388D1 cells as described previously (13), and was a gift from Dr. S. Durum, Yale University.

Assays

All assays were performed in Click's medium containing 5% fetal calf serum (FCS).

T cell proliferation. 2×10^4 cloned T cells were cultured in 0.2 ml in the presence or

absence of 2×10^5 whole spleen cells inactivated by mitomycin C (Sigma Chemical Co.) (30 min, 37°C, 50 μg/ml) and various additions. Cultures were harvested after 65 h after a 16-h (6.7 Ci/mmol) [^{3}H]thymidine ([^{3}H]TdR) pulse of 1 μCi/culture (New England Nuclear, Boston, MA).

T-dependent B cell proliferation. Cloned T cells were inactivated with mitomycin C and added to B cells as described previously (14). B cells were prepared by treatment of whole spleen cells with a rat monoclonal antibody (Y19) produced by Dr. B. Jones, Yale University, that reacts with the Thy-1 molecule (15), followed by rabbit complement. Proliferation was measured by a 4-h [^{3}H]TdR pulse of 1 μCi/culture after a 48-h culture period.

Fluorescence-activated Cell Sorter (FACS) Analysis

Purified monoclonal antibody 3D3 was coupled with biotin by the method of Bayer et al. (16) and fluoresceinated avidin (Vector Laboratories, Inc., Burlingame, CA) was used as a second-step reagent. All staining was performed at 4°C in PBS containing 2% FCS plus 0.1% sodium azide. Analysis was performed on a FACS IV machine (B-D FACS Systems, Sunnyvale, CA) with a logarithmic scale. 20,000 cells were routinely analyzed.

Iodination of Cell Surface Proteins

Cloned T cells were harvested, purified over lymphocyte separation medium (LSM) (Litton Bionetics Inc., Kensington, MD), and surface iodinated by a modification of the method of Cone and Marchalonis (17). 3×10^7 cells were suspended in 150 μl PBS to which 10 μl of 0.5 M sodium phosphate buffer, pH 7.0, 50 μl of 2 mg/ml lactoperoxidase (Sigma Chemical Co.), and 1 mCi Na^{125}I (100 mCi/ml; New England Nuclear) was added. The reaction was initiated by the addition of 20 μl of 0.03% H_2O_2 in PBS and incubated for 4 min at 30°C. An additional 20 μl of H_2O_2 was then added and the incubation continued at room temperature for another 10 min. To terminate the reaction, cells were washed once in ice-cold PBS containing 2 mM KI and 0.1% sodium azide, and then two more times in the same but with the addition of 2% FCS.

Biosynthetic Labeling of Cell Proteins

Viable cells were isolated over LSM and resuspended at 1×10^7 cells/ml in methionine- or cysteine-free RPMI 1640 supplemented with 2 mM glutamine, 60 μM 2-mercaptoethanol, and 5% FCS dialyzed against PBS. [^{35}S]methionine or [^{35}S]cysteine (Amersham Corp., Arlington Heights, IL) was added to 250 μCi/ml and the cells were incubated at 37°C. The incubation was terminated with the addition of ice-cold PBS containing 0.1% sodium azide and 2 mg/ml unlabeled amino acid and the cells were washed twice in the same. For pulse-chase experiments, the cells were preincubated in warmed, amino acid-free medium for 15 min at 37°C before the addition of label to deplete intracellular pools. Cells were then labeled for 15 min and the incubation terminated as above or, to chase, washed twice in methionine-containing medium plus 5% FCS and resuspended in the same for a further incubation at 37°C.

Immunoprecipitation

Labeled cells were resuspended in lysis buffer (see below) and incubated on ice for 15 min. The cell lysate was centrifuged at 100,000 *g* for 20 min in a Beckman airfuge (Beckman Instruments, Inc., Fullerton, CA) and the supernatant collected and precleared with 75 μl of MIg coupled to Sepharose per milliliter of lysate for 40 min at 4°C. The MIg-Sepharose was removed by centrifugation and 20 μl of antibody-coupled Sepharose (Pharmacia Fine Chemicals) (3 mg antibody/ml wet cyanogen bromide-activated Sepharose) was added per 0.5 ml of lysate. This mixture was incubated on a rotator at 4°C for 1–2 h after which the Sepharose beads were washed extensively in ice-cold lysis buffer. Bound material was eluted with the appropriate sample buffer as described below. To prevent nonspecific binding to the antibody-coupled Sepharose, the beads were blocked with FCS, washed in lysis buffer, and further blocked with an unlabeled lysate prepared from P3X63-Ag8.653 cells, a non–Ig-producing B cell hybridoma (18), before use.

Immunoprecipitations were performed with 3D3-coupled Sepharose or, as a control, Y3P monoclonal anti-Ia antibody (19) coupled to Sepharose.

One-dimensional Gel Analysis

Labeled cells were lysed in PBS containing 1% Nonidet P-40 (NP-40) (Particle Data, Inc., Elmhurst, IL), 0.5% deoxycholate sodium salt, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride. After immunoprecipitation, bound material was eluted with 50 μl SDS sample buffer (2.3% SDS/10% glycerol/62.5 mM Tris-HCl, pH 6.8) with or without 5% 2-mercaptoethanol and boiled for 3 min. Laemmli discontinuous SDS-PAGE was performed on a 10% polyacrylamide slab gel as described by O'Farrell (20). Molecular weights were estimated from the mobilities of ^{14}C-labeled markers (New England Nuclear) (cytochrome *c*, 12,300; carbonic anhydrase, 30,000; ovalbumin, 46,000; bovine serum albumin, 69,000; phosphorylase B, 97,400).

Two-dimensional Gel Analysis

Labeled cells were lysed in 0.5% NP-40/0.15M NaCl/0.02% sodium azide/1 mM phenylmethylsulfonyl fluoride/10 mM Tris-HCl, pH 7.2. After immunoprecipitation, antibody-Sepharose–bound material was eluted with 50 μl of isoelectric focusing sample buffer (9.5 M urea/2% NP-40/1.6% pH 5–7, 0.4% pH 3.5–10 Ampholines [LKB Instruments, Inc. Gaithersburg, MD] with or without 5% 2-mercaptoethanol). Nonequilibrium pH gradient electrophoresis followed by Laemmli discontinuous SDS-PAGE was performed by the method of O'Farrell et al. (21). Gradients routinely ranged from pH 4 to 8, and samples were electrophoresed for 3,000 volt-hours in the first dimension. Molecular weights were determined as above, with markers impregnated into tube gels and run alongside the sample gel in the second dimension.

Results

Monoclonal Antibody 3D3 Specific for Clone D10.G4.1 Induced D10.G4.1 Proliferation and D10.G4.1-dependent B Cell Proliferation. We have previously shown (2) that antisera raised against the cloned helper T cell line D10.G4.1 induces both proliferation of the cloned T cells, in the presence of accessory cells or an IL-1-rich supernatant, and cloned T cell-dependent B cell proliferation. Fig. 1 demonstrates that both these activities can also be induced by the anti-D10 monoclonal antibody 3D3. As was demonstrated for anti-D10 antisera using a monoclonal anti-Fc receptor antibody (2), the 3D3-induced T-dependent B cell proliferation is not Fc receptor dependent (data not shown). In addition, this monoclonal antibody induces T clone-dependent polyclonal B cell immunoglobulin secretion (22).

Results obtained with crude hybridoma supernatants suggested that very low concentrations of soluble monoclonal antibody were sufficient to activate these cloned T cells. We quantitated this finding using protein A-Sepharose affinity-purified antibody obtained from hybridoma culture supernatants. The experiment shown in Fig. 1 also demonstrates that soluble monoclonal antibody at a concentration as low as 1 ng/ml (10^{-11} M) is a potent activator of D10.G4.1 cells.

An Fab Fragment of Monoclonal Antibody 3D3 Blocks Specific but not Nonspecific Activation of D10.G4.1 Cells. The ability of antireceptor antibodies to mimic the action of ligand has been described in a number of hormonal systems (23, 24) and has been studied extensively with B cells using anti-Ig antisera (reviewed in reference 25). This activation is often found to require cross-linking of the receptors on the target cell surface. To determine the role of cross-linking of putative antigen receptors in the activation of T cells, an Fab fragment of

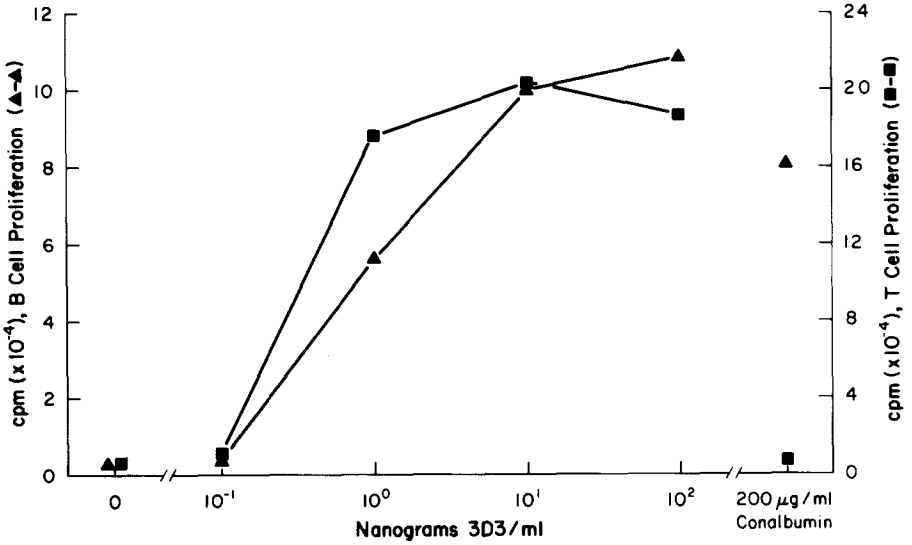

FIGURE 1. Titration of monoclonal antibody 3D3 induction of D10.G4.1 proliferation and D10.G4.1-dependent B cell proliferation. D10.G4.1 proliferation (■) measured in a 65-h assay containing 2×10^4 cloned T cells, 1% of a partially purified, IL-1-rich P388D1 supernatant, and various concentrations of 3D3, or 200 μg/ml conalbumin. B cell proliferation (▲) was measured in a 48-h assay containing 2×10^4 mitomycin C-treated D10.G4.1 cells, 2×10^5 BALB.K B cells (anti-Thy-1 plus complement-treated spleen), and various concentrations of 3D3, or 200 μg/ml conalbumin. Data are expressed as the mean cpm [^{3}H]TdR incorporation of triplicate cultures.

monoclonal antibody 3D3 was prepared by papain digestion. Although very low concentrations of the intact monoclonal antibody could induce proliferation of the cloned T cells in the presence of an IL-1-rich supernatant, even very high concentrations of the Fab fragment had little effect (Fig. 2, *right*). However, the addition of a cross-linking reagent, rabbit anti-MIg, restored the response of as little as 3 ng/ml of the Fab fragment (Fig. 2, *right*). Therefore, the inability of the Fab fragment to activate D10.G4.1 cells was not due to failure of the Fab fragment to bind the T cell surface, but rather to the change in valency.

Because the Fab fragment itself did not activate, we were able to test its ability to inhibit the activation of D10.G4.1 cells. The experiment shown in Fig. 2 (*left*) shows that the Fab fragment of 3D3 could completely inhibit D10.G4.1 proliferation induced by the intact monoclonal antibody. In addition, the Fab fragment inhibited, by 86% at 30 μg/ml, antigen-induced T cell proliferation in the presence of a source of syngeneic antigen-presenting cells. D10.G4.1 recognizes conalbumin in the context of I-A^k and is also alloreactive to I-A^b (2).[2] The Fab fragment also inhibited, by at least 90%, D10.G4.1 activation by allogeneic Ia as measured by the proliferative response to H-2^b spleen cells (Fig. 2, *left*). Thus a single monoclonal antibody blocks activation both by antigen and self-Ia, and by allogeneic Ia, strongly suggesting that both these ligands can be recognized by a single receptor molecule. The fact that ~1,000-fold higher concentrations of the

[2] Kaye, J., S. Gillis, S. B. Mizel, E. M. Shevach, T. R. Malek, C. A. Dinarello, and C. A. Janeway, Jr. Growth of a cloned helper T cell line induced by a monoclonal antibody specific for the antigen receptor. Interleukin 1 is required for the expression of receptors for interleukin 2. Manuscript submitted for publication.

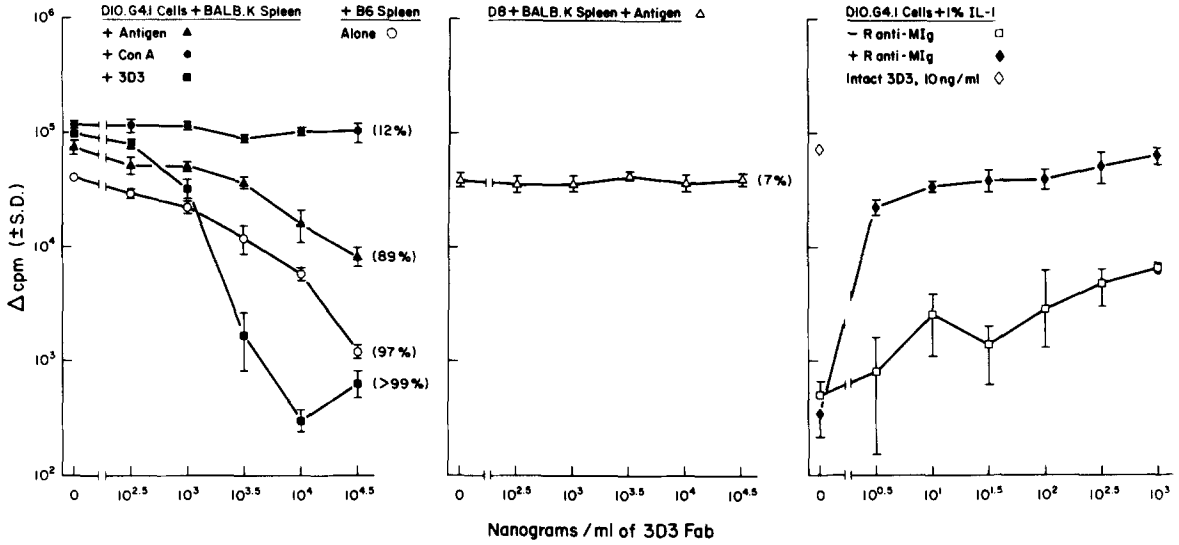

FIGURE 2. An Fab fragment of monoclonal antibody 3D3 blocks specific but not nonspecific activation of D10.G4.1 cells. (*Right*) D10.G4.1 proliferation was measured in a 65-h assay containing 2×10^4 cloned T cells, 1% of a partially purified, IL-1-rich P388D1 supernatant, and various concentrations of a 3D3 Fab fragment, in the presence (♦) or absence (□) of 10 μg/ml affinity-purified rabbit anti-MIg antibody. For comparison, D10.G4.1 proliferation in the presence of 10 ng/ml intact 3D3 and 1% IL-1 is shown (◇). (*Left*) D10.G4.1 proliferation was measured in an assay containing 2×10^4 cloned T cells, 2×10^5 mitomycin C-treated BALB.K ($H\text{-}2^k$) spleen cells, various concentrations of a 3D3 Fab fragment, and 200 μg/ml conalbumin (▲), 2.5 μg/ml concanavalin A (●), or 10 ng/ml intact 3D3 (■). Alternately, D10.G4.1 proliferation was assayed in the presence of 2×10^5 mitomycin C-treated C57BL6 ($H\text{-}2^b$) spleen cells and various concentrations of a 3D3 Fab fragment (○). (*Middle*) D8 proliferation was assayed in a 65-h assay containing 2×10^4 cloned T cells, 2×10^5 mitomycin C-treated BALB.K spleen cells, 200 μg/ml ovalbumin, and various concentrations of a 3D3 Fab fragment (△). In parenthesis is the percent inhibition of the response for cultures containing 30 μg/ml ($10^{4.5}$ μg/ml) Fab fragment. All data are expressed as the mean Δcpm [^{3}H]TdR incorporation of triplicate cultures ± SD.

Fab fragment were necessary to block activation by 50% than to induce half-maximal activation in the presence of anti-MIg suggests that very few receptor molecules per cell need be engaged to trigger the cell. Table I lists the Fab fragment concentrations that cause 50% inhibition of D10.G4.1 proliferation induced by various means. (Exp. 1 is a summary of Fig. 2 and Exp. 2 is an independent experiment using a different preparation of 3D3 Fab fragment.) Note that although Exp. 1 and Exp. 2 differ in the actual concentrations of Fab fragment able to inhibit responses by 50%, the relative concentration of Fab required for 50% inhibition is nearly constant.

In contrast, the Fab fragment does not inhibit D10.G4.1 proliferation induced by concanavalin A (Fig. 2, *left* and Table I) or RaMBr (Table I). We have previously reported (2) that monoclonal antibody 3D3 does not activate another helper T cell clone, D8, cloned from the same donor cells as D10.G4.1 but with specificity for ovalbumin in the context of $I\text{-}A^k$. As expected, the 3D3 Fab fragment does not inhibit the proliferation of D8 cells induced by antigen and syngeneic accessory cells (Fig. 2, *middle* and Table I).

3D3 Precipitates a Disulfide-linked Heterodimer from D10.G4.1 Cells. Immu-

TABLE I

3D3 Fab Fragment Blocks Specific Activation of D10.G4.1

T cell clone*	Stimulus‡	Concentration of 3D3 Fab fragment needed to inhibit response by 50%			
		Exp. 1§	(ratio)‖	Exp. 2	(ratio)
		μg/ml			
D10.G4.1	3D3 + BALB.K	0.7	(1.0)	1.8	(1.0)
D10.G4.1	C57BL/6J	1.3	(1.9)	3.8	(2.1)
D10.G4.1	Conalbumin + BALB.K	2.8	(4.0)	9.0	(5.0)
D10.G4.1	Con A + BALB.K	>30¶		>30	
D10.G4.1	RaMBr + BALB.K	ND**		>30	
D8	Ovalbumin + BALB.K	>30		>30	

* 2×10^4 cloned T cells/200 μl culture.

‡ 3D3, 10 ng/ml; conalbumin and ovalbumin, 200 μg/ml; concanavalin A, 2.5 μg/ml; RaMBr, 1:100; BALB.K ($H\text{-}2^k$), C57BL/6J ($H\text{-}2^b$), 2×10^5 mitomycin C-treated spleen cells/200 μl culture.

§ Exp. 1 and Exp. 2 use independently prepared 3D3 Fab fragments.

‖ Relative amount of 3D3 Fab required to cause 50% inhibition of response to the stimulus shown; concentration of 3D3 Fab required to inhibit by 50% stimulation by 3D3 + BALB.K spleen cells is 1.0.

¶ No inhibition observed at highest concentration tested.

** Not done.

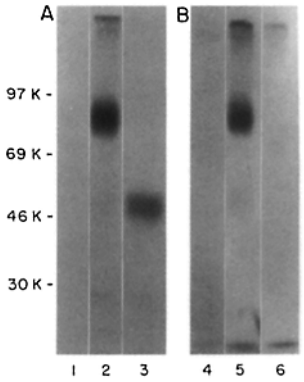

FIGURE 3. Monoclonal antibody 3D3 immunoprecipitates a disulfide-linked dimer from surface-labeled D10.G4.1 cells but not from D8 cells. Material isolated from detergent lysates of surface-iodinated D10.G4.1 cells (lanes 1–5) or D8 cells (lane 6) by immunoprecipitation with 3D3 (lanes 2, 3, 5, 6) or a control monoclonal antibody (Y3P) (lanes 1, 4), was analyzed by SDS-PAGE under nonreducing conditions (lanes 1, 2, 4–6) or reducing conditions (lane 3). Lanes 1–5 are immunoprecipitates from 1×10^7 cells; lane 6 from 1.5×10^7 cells. D8 was labeled to a higher specific activity (4.2×10^4 trichloroacetic acid (TCA) precipitable, cpm/10^5 cells) than was D10.G4.1 (2.6×10^4 TCA precipitable, cpm/10^5 cells) in Exp. B.

noprecipitation with 3D3 from lactoperoxidase-catalyzed, surface-iodinated D10.G4.1 cells followed by SDS-PAGE analysis under reducing conditions reveals a single broad band with an apparent molecular weight of 48,000 (Fig. 3, lane 3). Under nonreducing conditions, the immunoprecipitated material has an apparent molecular weight of 83,000, suggesting that the native molecule is found on the cell surface as a disulfide-linked dimer (Fig. 3, lane 2). The discrepancy between the mobilities of the nonreduced and reduced molecules in SDS-PAGE, relative to the expected mobilities for molecules differing twofold

in mass, may be the result of intrachain disulfide bonds that prevent complete denaturation of the nonreduced molecule in SDS. Alternately, noncovalent interactions between the reduced monomers might result in a slower mobility than expected, a possibility supported by molecular weight determinations obtained by two-dimensional gel analysis. The addition of iodoacetamide to the lysis buffer did not alter this molecular weight profile (data not shown), and it is therefore unlikely that the higher molecular weight form found under nonreducing conditions is an artifactual dimer formed by reactions between free sulfhydryl groups.

As discussed above, 3D3 does not activate the helper cell clone D8, although a clonotypic antiserum can be raised against these cells (2). In addition, no binding of 3D3 to D8 cells can be detected by immunofluorescence and FACS analysis (Fig. 4, *left*), while 3D3 stains all cells of clone D10.G4.1 (Fig. 4, *right*). As expected, there are no detectable bands in 3D3 immunoprecipitates from surface-iodinated D8 cells (Fig. 3, lane 6).

Nonequilibrium pH gradient electrophoresis followed by SDS-PAGE, both under reducing conditions, demonstrates that the material precipitated from surface-iodinated D10.G4.1 cells is a heterodimer comprised of an acidic and a basic chain, each with a apparent 42,000 mol wt (Fig. 5*A*). The basic chain, and to a lesser degree the acidic chain, displayed microheterogeneity in the first dimension, suggesting the possibility of multiple glycosylated forms. When analyzed by two-dimensional gel electrophoresis under nonreducing conditions, this identical sample runs with an intermediate pI, forming a clonotype of spots in the first dimension at an apparent molecular weight of 80,000, confirming the α:β structure of the dimer (Fig. 5*B*). Note also the presence of material that appears to have been reduced in the second dimension, despite the absence of added reducing agents. This pattern is consistent with a mild partial reduction of the sample. It is unlikely that the 42,000 mol wt material in Fig. 5*B* represents a proteolytic cleavage of the dimer because, as shown in Fig. 5*A*, the reduced subunits are intact. Analysis of 3D3 immunoprecipitated material by two-dimensional gel electrophoresis under nonreducing vs. reducing conditions (Fig. 5 and data not shown) suggests that only a fraction of the total amount of nonreduced

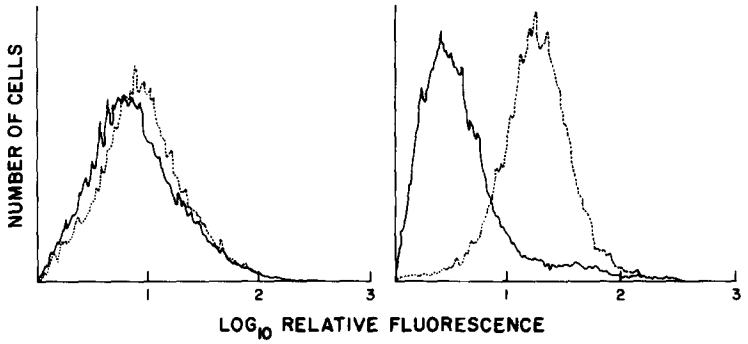

FIGURE 4. Monoclonal antibody 3D3 stains D10.G4.1 cells but not D8 cells. D8 cells (*left*) or D10.G4.1 cells (*right*) were stained with (---) or without (——) biotinylated 3D3 followed by fluorescein isothiocyanate-avidin and analyzed by FACS.

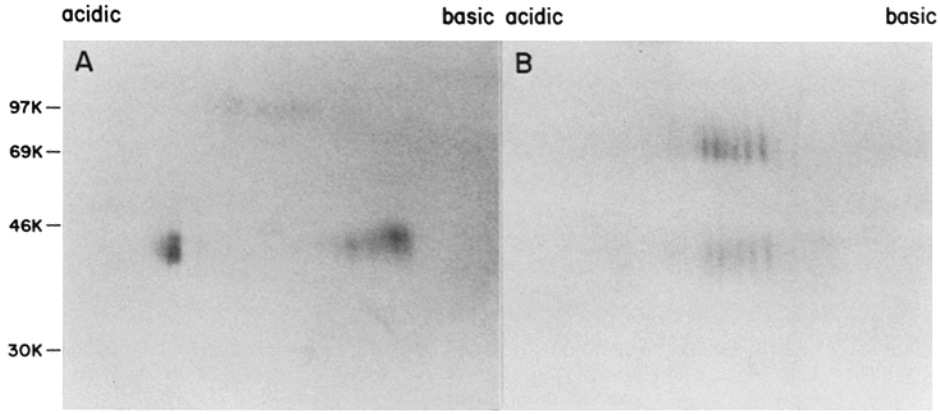

FIGURE 5. The dimer isolated from surface-labeled D10.G4.1 cells with monoclonal antibody 3D3 is comprised of an acidic and a basic subunit. D10.G4.1 cells were lactoperoxidase catalyzed surface iodinated, lysed in NP-40, immunoprecipitated with 3D3, and analyzed by nonequilibrium pH gradient electrophoresis followed by SDS-PAGE on a 10% polyacrylamide slab gel under reducing (*left*) or nonreducing (*right*) conditions.

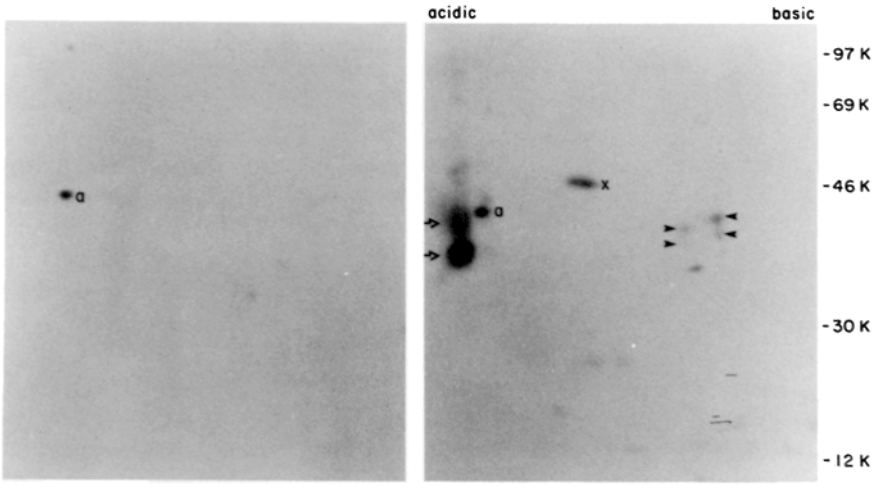

FIGURE 6. Identification of internal and surface forms of the heterodimer reactive with monoclonal antibody 3D3 from biosynthetically labeled D10.G4.1 cells. D10.G4.1 cells were labeled with [^{35}S]methionine for 4 h, lysed in NP-40, and immunoprecipitated with a control monoclonal antibody (Y3P) (*left*) or 3D3 (*right*). Immunoprecipitates were analyzed under reducing conditions as in Fig. 5. Acidic (open arrows) and basic components (closed arrows) of the heterodimer are indicated. Actin (a) has been identified based on electrophoretic mobility in both dimensions, and X denotes material believed to be nonspecifically precipitated in this experiment.

heterodimer immunoprecipitated may actually enter the gel. It is possible that some aggregation is occuring in the absence of SDS in the first dimension.

The acidic and basic chains were also immunoprecipitated from D10.G4.1 cells biosynthetically labeled with either methionine (Fig. 6) or cysteine (Fig. 7), although the basic chain labeled poorly under these conditions. The material designated "X" in Fig. 6 is an inconsistent finding, often found in control precipitates, and is therefore believed to be nonspecifically precipitated, possibly

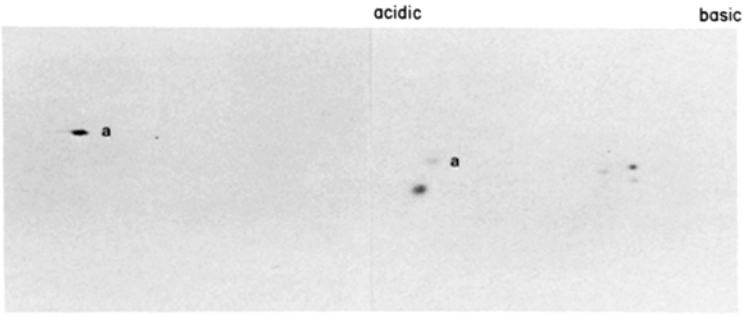

FIGURE 7. Two-dimensional gel analysis of a 3D3 immunoprecipitate from pulse-labeled D10.G4.1 cells. D10.G4.1 cells were labeled for 15 min with [^{35}S]cysteine, lysed in NP-40, and immunoprecipitated with a control monoclonal antibody (Y3P) (*left*) or 3D3 (*right*), and analyzed by two-dimensional gel electrophoresis under reducing conditions as in Fig. 5. Actin (a) is indicated.

due to its affinity for immune complexes. Note that the basic chain appears as a number of spots differing in apparent molecular weight as well as charge. Whether these proteins represent posttranslational modifications of a single polypeptide or multiple polypeptides remains to be determined (see below). The acidic chain can be seen to have a strongly labeled component of 38,000 mol wt (actually a doublet) and a less strongly labeled higher molecular weight component of 42,000 (Fig. 6).

To determine the relationship of the multiple forms of the two chains and identify their biosynthetic precursors, we performed the pulse-chase experiment shown in Fig. 8. A series of acidic spots, 35,000–38,000 mol wt, whose acidity decreased with the increase in molecular weight, are detectable in 3D3 immunoprecipitates from D10.G4.1 cells after a 15-min pulse with labeled methionine (Fig. 8, open arrows). After a 2-h chase with cold methionine, the three lowest molecular weight forms were no longer detectable, more material chased into the higher molecular weight form of the 38,000 mol wt doublet, and 42,000 mol wt material began to appear. After 4 h of chase, the 42,000 mol wt spot was more pronounced and more acidic forms became detectable. The two-dimensional gel pattern obtained by 3D3 immunoprecipitation from D10.G4.1 cells after a 15-min pulse with labeled cysteine was identical to that seen with methionine: a 38,000 mol wt form of the acidic chain and faintly labeled, lower molecular weight and more acidic forms (Fig. 7). Note that the basic chain again appears as multiple spots in this experiment. Due to the complexity of these results, an exact sequential pathway for the biosynthesis of the acidic chain cannot be determined. However, it appears that the acidic chain undergoes a series of posttranslational modifications resulting in 38,000 and 42,000 mol wt forms, the latter most likely representing the mature cell surface polypeptide, since its pattern is essentially identical to the material precipitated after surface iodination (Fig. 5), and the former most likely representing the major pool of internal polypeptide. In addition, these experiments suggest that the primary biosynthetic precursor of the acidic chain may have an apparent molecular weight as low as 35,000. A preliminary analysis under nonreducing conditions after a short pulse with labeled methionine suggests that the lower molecular weight forms of the

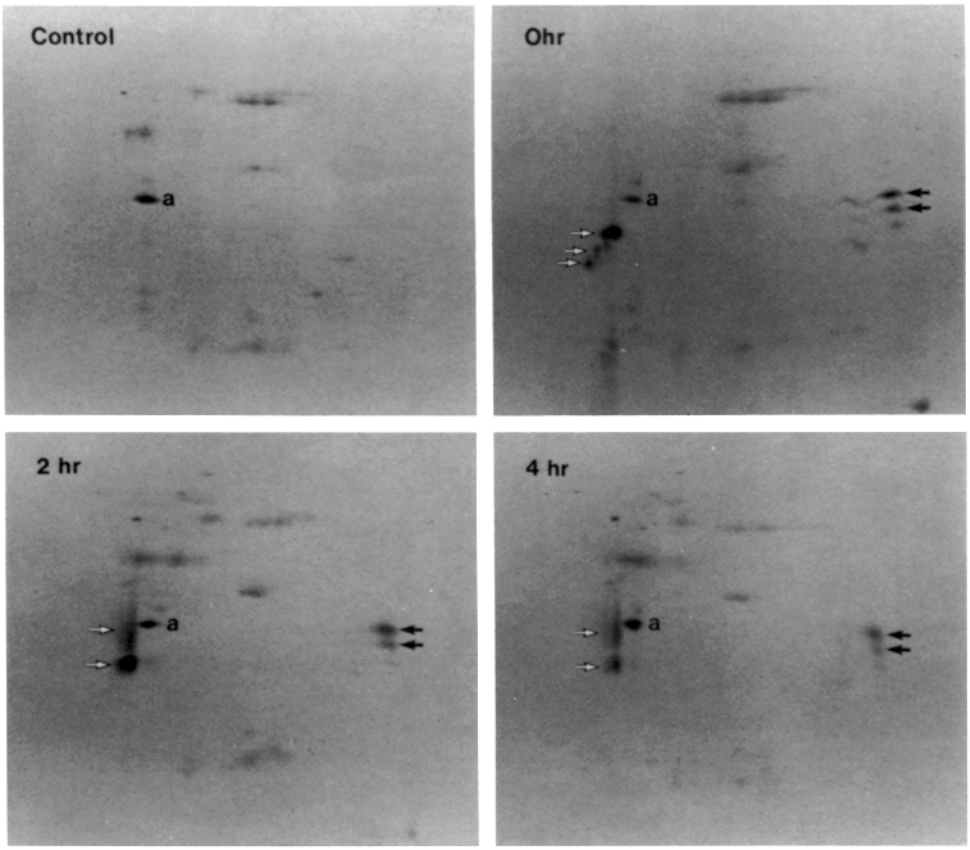

FIGURE 8. Posttranslational processing of a heterodimer isolated from D10.G4.1 cells with monoclonal antibody 3D3. D10.G4.1 cells were labeled for 15 min with [^{35}S]methionine, lysed in NP-40, and immunoprecipitated with a control antibody (Y3P) (*top left*) or 3D3 (*top right*) and analyzed by two-dimensional gel analysis under reducing conditions as in Fig. 5. After the pulse-label, some cells were washed into unlabeled methionine-containing medium and incubated for an additional 2 h (*bottom left*) or 4 h (*bottom right*) before immunoprecipitation with 3D3. Acidic components (open arrows) and basic components (closed arrows) of the heterodimer are indicated, as is actin (a).

acidic chain are already complexed with the basic chain (data not shown). Because we do not know whether 3D3 can immunoprecipitate the acidic chain before it has complexed with the basic chain, it is possible that earlier biosynthetic precursors of the acidic chain exist. Note that there is little difference in the appearance of the basic chain during the pulse-chase (Fig. 8, closed arrows), suggesting either less posttranslational modification or a more rapid processing and entry into heterodimers than that of the acidic chain.

Discussion

This report describes the characteristics of a monoclonal antibody directed against a cloned helper T cell line, both in terms of its ability to specifically activate these cells and, in monovalent form, to block specific activation by antigen. This antibody also enabled us to structurally characterize the molecule,

presumably the antigen/Ia receptor, responsible for the observed biological activities of the antibody. We demonstrated that very low concentrations of soluble monoclonal antibody (1–10 ng/ml) induce both T cell proliferation (in the presence of IL-1) and T-dependent B cell proliferation. Thus, as reported previously (2), this antibody mimics antigen and Ia in its ability to activate both D10.G4.1 cells and B cells in a D10.G4.1-dependent manner.

However, an Fab fragment of this antibody, even at 1,000-fold higher concentrations, had no biological activity when either T cell proliferation or IL-2 production (data not presented) was measured. When cross-linked by anti-MIg antibody, the ability of the Fab fragment to induce D10.G4.1 proliferation was restored. This implies that cross-linking of antigen receptors, and not receptor occupancy per se, provides the relevant signal for activation in this system. It is not known how many 3D3 determinants a single receptor molecule possesses and therefore the extent of cross-linking necessary for activation cannot be determined. The possibility that two different monoclonal antireceptor antibodies are present in 3D3 has been ruled out both by extensive recloning without change in biological activity and by the demonstration of a single species of antibody by two-dimensional gel electrophoresis (2 and J. Kaye, unpublished results). It is possible that allogeneic Ia or antigen and self-Ia presented as an array on an antigen-presenting cell surface also acts as a multivalent cross-linker of T cell antigen receptors. Two other laboratories (26–28) have described monoclonal antibodies directed against clonotypic molecules on cloned helper or cytotoxic T cells which inhibit antigen-specific functions in soluble form but induce IL-2 secretion or proliferation when polymerized on a solid support. These observations also support the concept of receptor cross-linking as the trigger for T cell activation. The unique ability of minute amounts of soluble monoclonal antibody 3D3 to activate D10.G4.1 cells may be due to an intrinsic property of the cloned T cells themselves (for example, the number of receptors or threshold of activation) or to the particular determinant recognized by 3D3 that allows efficient cross-linking of antigen receptors. It is unlikely that D10.G4.1 is being activated by aggregates of 3D3, given the isotype of the antibody (IgG1) and the finding that very high dilutions of the primary 3D3 hybridoma culture supernatant (2), as well as low concentrations of purified monoclonal antibody, are stimulatory.

The inability of the Fab fragment of 3D3 to activate D10.G4.1 cells allowed us to investigate the effects of this antibody on the induction of proliferation by antigen. D10.G4.1 proliferation induced by conalbumin and I-A^k or by I-A^b alone was inhibited ~90% by the Fab fragment. The simplest interpretation of this finding is that both allogeneic Ia and antigen plus self-Ia are recognized by the same receptor molecule. However, these experiments do not address the question of whether one or more separate binding sites exist for these ligands. A comparison of the concentrations of Fab fragment necessary to inhibit D10.G4.1 activation by 50% (Table I) results in a hierarchy of ease of inhibition. The relatively low concentration of Fab fragment necessary to inhibit the intact 3D3-induced response, as compared with allogeneic Ia or antigen plus self-Ia–induced responses, is consistent with the finding that Ia recognition, and possibly all cell contact, is not necessary for 3D3-dependent activation.[2] We were also

intrigued by the finding that the response to I-A^b required a lower concentration of the Fab fragment to obtain 50% inhibition than did the antigen response. It is possible that the affinity of the D10.G4.1 receptor is lower for I-A^b than for I-A^k plus conalbumin. It is tempting to speculate that alloreactivity may often be the result of a low affinity cross-reaction per receptor site but, given that cross-linking of receptors may be involved in triggering and that the number of identical allogeneic Ia molecules on an allogeneic antigen-presenting cell is surely greater by orders of magnitude than the number of appropriate antigen/Ia moieties on a syngeneic antigen-presenting cell, this weak cross-reaction results in a high avidity of interaction and, in turn, activation. It should be noted that the response of D10.G4.1 cells to I-A^b relative to the response to conalbumin and I-A^k is the same whether cultured in serum-free medium or normal mouse serum- or FCS-containing medium. It is therefore unlikely that this alloreactivity is due to the recognition of an FCS protein in the context of allogeneic Ia, as has been suggested in a recent report (29). Mitogen- or RaMBr-induced activation was not influenced by the presence of the Fab fragment, demonstrating that the observed inhibition was specific. In addition, the activation of another helper T cell clone by antigen was not inhibited by the Fab fragment. Thus the Fab fragment exhibits the same specificity of reactivity as was demonstrated for intact 3D3 in functional, immunofluorescence, and immunoprecipitation studies.

Immunoprecipitation analysis with 3D3 from surface or biosynthetically labeled D10.G4.1 cells identified an 83,000 mol wt heterodimer as the target antigen. This molecule exhibited microheterogeneity by charge and is therefore most likely a glycoprotein. Tunicamycin inhibition of posttranslational modification appears to confirm this (Kaye and Janeway, unpublished observations). The subunits of the dimer have similar molecular weights as determined by SDS-PAGE, but can be distinguished by isoelectric focusing. Structurally similar molecules have been isolated from cloned human and murine cell lines in other laboratories, also by using monoclonal antibodies that inhibit specific activation (3–5, 7, 27, 28, 30). Pulse-chase experiments demonstrated that the acidic subunit of this dimer undergoes a series of posttranslational modifications resulting in 38,000 and 42,000 mol wt polypeptides. The former appears to be part of a sizeable internal pool of heterodimers and the latter, the cell surface subunit. Because the pulse-chase experiments performed to date have not revealed a simple sequential transition between all the forms detected, the exact biosynthetic pathway cannot be determined. However, the primary biosynthetic precursor of the acidic subunit appears to have a molecular weight as low as 35,000, and may be even smaller (Kaye and Janeway, unpublished observation). These experiments were uninformative concerning the biosynthesis of the basic chain for reasons that are as yet unclear.

In summary, we have used a monoclonal source of functionally defined cells and a monoclonal antibody to investigate both the specific requirements for cell activation and the biochemical nature of the molecule involved. Details of the structure of this molecule, its relationship to other surface molecules involved in cell interactions, and the regulation of its biosynthesis should provide further linkage between structural and functional approaches to understanding T cell biology.

Summary

We characterize a monoclonal antibody directed against the antigen/Ia receptor of a cloned helper T cell line that induced T cell clone proliferation and T cell clone-dependent B cell proliferation at antibody concentrations as low as 10^{-11} M. An Fab fragment of this antibody was not stimulatory, implicating cross-linking of antigen receptors as the primary signal for T cell activation. The Fab fragment inhibited activation of this clone by both allogeneic Ia and antigen plus self-Ia, but not by the nonspecific stimulators concanavalin A and rabbit anti-mouse brain serum. This strongly supports the hypothesis that a single molecule mediates both self-Ia plus antigen and non–self-Ia recognition. This molecule is presumably the disulfide-linked heterodimer comprised of 42,000 mol wt acidic and basic subunits precipitated by this monoclonal antibody. The cell surface and internal precursor forms of this protein are also identified. In addition, the response to allogeneic Ia stimulation was more readily inhibited by the Fab fragment than was the response to antigen plus self-Ia, suggesting that alloreactivity reflects a low affinity interaction with a ligand represented at high frequency on the stimulatory cell.

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