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Immunology

Antigen presentation by supported planar membranes containing affinity-purified I-A^d

(histocompatibility antigens/peptide antigens/T-cell hybridoma/L3T4 antigen/lipid bilayers)

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ABSTRACT **I-A^d, purified from A20-1.11 cells by affinity chromatography, was incorporated into supported planar membranes by incubation of I-A^d-containing phospholipid vesicles with clean glass coverslips. Such planar membranes present a peptide digest of ovalbumin to the ovalbumin-specific, I-A^d-restricted T-cell hybridoma 3DO-54.8, resulting in the antigen-specific release of interleukin 2. However, when the same material was provided in the form of small unilamellar vesicles, no response was obtained. Antigen presentation by the I-A^d-containing planar membranes was inhibited by the monoclonal antibody MKD6 (anti-I-A^d) but not by the antibody 10-2.16 (anti-I-A^k). The antibody GK1.5, which recognizes the T-cell surface antigen L3T4, was also inhibitory. In contrast to the results with purified I-A^d, crude membrane preparations from A20-1.11 cells were effective in antigen presentation in both planar and vesicular forms.**

Ia antigens are genetically polymorphic cell surface glycoproteins encoded by the I region of the murine major histocompatibility complex. Helper T cells respond to foreign antigens only when they are recognized in conjunction with the appropriate I-region molecule on the surface of the antigen-presenting cell (1–3). For globular protein antigens, it has been shown that it is usually not the native structure, but a peptide fragment that is recognized by the T cell (4, 5). However, there is no direct evidence indicating whether T-cell receptor binds to a complex of Ia and foreign antigen or whether they are recognized independently. Furthermore, there is conflicting evidence regarding whether foreign antigens can interact with either Ia (6) or T-cell receptor alone (7) or whether it is necessary for all three components to form a termolecular complex to obtain significant interaction (8). It is also unclear whether Ia antigen and peptide are sufficient for T-cell stimulation or whether other cell surface antigens are necessary for the formation of T-cell antigen-presenting cell conjugates.

One approach to understanding the cell surface recognition events that lead to the stimulation of T cell help is to use purified Ia antigens reconstituted into model membranes. Recent work in this laboratory has been directed toward the use of supported planar membranes, suitable for fluorescence microscopy, in the study of cell surface recognition events (9). Brian and McConnell (10) have described a simplified method for incorporating purified major histocompatibility proteins into such supported membranes.

In this report, we describe the incorporation of purified I-A^d into planar membranes and the ability of these membranes to present processed antigen to the T-cell hybridoma 3DO-54.8 (5). It has been shown previously that this hybridoma releases the lymphokine interleukin 2 (IL-2) when ovalbumin is presented by I-A^d-bearing B-cell lymphomas and that it responds to cyanogen bromide and tryptic fragments of ovalbumin, but not to native ovalbumin, when presented by glutaraldehyde-fixed cells (5). In addition, we show that antigen presentation by planar membranes is inhibitable both by anti-I-A^d antibody and by the antibody GK1.5 (11), which binds to the non-polymorphic T-cell antigen L3T4. Previously, the ability of GK1.5 antibody to inhibit T-cell stimulation has been correlated with the involvement of Ia in the recognition event (12, 13).

MATERIALS AND METHODS

Cell Lines, Mice, and Culture Media. Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 μg of penicillin per ml, and 100 μg of streptomycin per ml. For the cell lines A20-1.11 (14), 3DO-54.8 and the IL-2-dependent line CTLL (15), the medium was supplemented with 50 μM 2-mercaptoethanol and 100 μM nonessential amino acids (GIBCO). In addition, CTLL cells were maintained in 10% (vol/vol) supernatant from Con A-stimulated rat spleen cells (16). CH1 cells were grown as ascites tumors in B10H-2^{a}H-4^{b}p/Wts mice, which were bred in our facilities from stock obtained from G. Haughton (University of North Carolina).

Antibodies and Affinity Columns. MKD6 antibody (I-A^d-specific) (17) and 10-2.16 antibody (I-A^k-specific) (18) were purified from culture supernatants by using protein A-Sepharose (Pharmacia). The purified antibodies were coupled at pH 8.3 to cyanogen bromide-activated Sepharose (SIGMA) at 2 mg of antibody per ml of resin. For experiments with GK1.5 (anti-L3T4) (11) and I21/7.7 (anti-LFA-1) (19), culture supernatants were used.

Fluoresceination of antibodies was carried out for 18 hr at 4°C in 0.1 M sodium carbonate/bicarbonate buffer, pH 9, at a fluorescein isothiocyanate/protein ratio of 10:1 (mol/mol). Fluorescent antibodies were purified on a 0.7 × 25 cm Sephadex G-25 column in phosphate-buffered saline (pH 7.2) (P_i/NaCl). A ratio of 4.5 fluorescein/antibody was obtained for the 10-2.16 antibody and a ratio of 2.5:1 was obtained for the MKD6 antibody.

I-A^d Purification. I-A^d was purified from A20-1.11 cells, grown *in vitro*. A whole cell lysate was prepared in 0.5% (wt/vol) Nonidet P-40 in 0.01 M Tris/0.14 M NaCl/0.02% NaN_3, pH 8.3 (Tris/NaCl/NaN_3) plus 5 μM phenylmethylsulfonyl fluoride as described by Turkewitz *et al.* (20). A glycoprotein pool was obtained from the whole cell lysate by chromatography on lentil lectin-Sepharose 4B (Pharmacia). The glycoproteins were eluted with 10% α-methyl-D-mannoside in 0.5% (wt/vol) sodium deoxycholate in Tris/NaCl/NaN_3. About 20 mg of glycoproteins was obtained from 10^{10} cells. In order to monitor recovery from the MKD6 affinity column, a 50-μg aliquot of glycoproteins was iodinated by

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Abbreviation: IL-2, interleukin 2.

the Iodogen (Pierce) method (21) using carrier-free ^{125}I (Amersham, 17.4 mCi/μg; 1 Ci = 37 GBq). The ^{125}I-labeled glycoproteins were combined with the bulk glycoprotein pool to give a specific activity of 1500 cpm/μg of total protein and the mixture was applied to a 2-ml MKD6 column. The column was washed with 20 vol of 0.5% (wt/vol) deoxycholate in Tris/NaCl/NaN_3 and then with 10 vol of 30 mM octyl glucoside in Tris/NaCl/NaN_3. The I-A^d was eluted with 2 M ammonium thiocyanate in octyl glucoside/Tris/NaCl/NaN_3. Some I-A^d could be eluted with 3 M NaCl in octyl glucoside, but elution was more efficient with ammonium thiocyanate. Extraction of an aliquot of the MKD6-Sepharose with 1% $NaDodSO_4$ gel buffer showed that no I-A^d remained after the thiocyanate treatment. Prior to reconstitution, I-A was dialyzed against 0.5% sodium deoxycholate, in Tris/NaCl/NaN_3, to remove salt. Sometimes, dilute fractions were further concentrated on a 0.5-ml lentil lectin column prior to reconstitution.

I-A^k was purified from a whole cell lysate of *in vivo* grown CH1 cells by lentil lectin chromatography followed by chromatography on 10-2.16 Sepharose 4B. I-A^k showed very similar elution properties to I-A^d.

Protein Concentrations. Protein concentrations were determined by the method of Lowry, using bovine serum albumin as a standard. I-A concentration was confirmed by using the *o*-phthalaldehyde test on acid-hydrolyzed I-A to determine total amino groups (as described in ref. 22). The results of the Lowry and *o*-phthalaldehyde tests agreed within 5%. Conversion from units of micrograms to cell equivalents of I-A was based upon the approximation that 567 μg represents 100% yield of I-A from 10^{10} cells (see *Results*). The amount of material incorporated into the planar membranes was calculated as described (10).

Preparation of Detergent-Solubilized Membrane Vesicles. A20-1.11 membranes were obtained by several cycles of freeze-thaw lysis in 0.015 M NaCl/5 mM sodium phosphate/0.02% NaN_3/5 μM phenylmethylsulfonyl fluoride, pH 7, at a cell density of 10^7 per ml. Debris was removed by low-speed centrifugation and the membranes were collected by pelleting at 95,000 × *g*, then resuspended to a concentration of 10^8 cell equivalents per ml (1–1.5 mg of membrane protein per ml) in 0.5% (wt/vol) sodium deoxycholate/Tris/NaCl/NaN_3, pH 8.0. The detergent-solubilized membranes were filter sterilized and dialyzed under sterile conditions to produce cell membrane vesicles.

Reconstitution. I-A^k and I-A^d were reconstituted with egg phosphatidylcholine and cholesterol (7:2 molar ratio; Sigma) by detergent dialysis as described for H-$2K^k$ (10). The lipid/detergent/protein suspension was filter sterilized through a 0.2-μm filter and dialyzed at 4°C for 72 hr against three changes of 1 liter of sterile P_i/NaCl followed by overnight dialysis against serum-free medium. The final lipid concentration was 0.2 mg/ml and the I-A concentration was 30 μg/ml, except where indicated otherwise. The preparation of planar membranes for fluorescence microscopy has been described (10).

Stimulation of IL-2 Release from 3DO-54.8 Cells. Experiments with I-A-containing lipid vesicles were carried out in 96-well culture dishes (Costar) as follows. Each well received 50 μl of vesicles, 50 μl of medium with 20% fetal calf serum, 50 μl of peptide digest, or 50 μl of serum-free medium followed by 100 μl of T cells (at a cell density of 10^6 per ml). After 24 hr at 37°C supernatants were removed and assayed for IL-2 as described below.

Planar membranes were prepared in 24-well culture dishes. One hundred microliters of liposomes plus 100 μl of serum-free medium were placed in a well and overlayed with a 12-mm (diameter) round cover glass (Rochester Scientific) that had been cleaned and ethanol sterilized as described (10). After 30 min, each well was filled to the brim with 3.3 ml of serum-free medium and the cover glass was turned over to expose the planar membrane; 3.2 ml of medium was then removed and 0.2 ml of medium containing 20% serum was added, followed by 100 μl of antigen or medium and then by 0.4 ml of 3DO-54.8 cells (at 10^6 per ml). For antibody inhibition, 10 μl of antibody in P_i/NaCl was added prior to the addition of T cells. At no time were the planar membranes exposed to air. After 24 hr, aliquots of supernatant were removed and assayed for IL-2.

IL-2 Assay. IL-2 was assayed by using the IL-2-dependent cell line CTLL. Eighty-microliter aliquots of 3DO-54.8 culture supernatants were combined with 20 μl of CTLL (5 × 10^5 per ml). After 24 hr, CTLL proliferation was measured by pulsing each well for 7 hr with 1 μCi of [*methyl*-^{3}H]thymidine (6.7 μCi/mmol; New England Nuclear). Cells were harvested and processed for scintillation spectroscopy. Visual scoring of CTLL viability was used to confirm results.

Miscellaneous Procedures. Citraconylation of ovalbumin was carried out in 6 M guanidine·HCl by the method of Gibbons and Perham (23). Trypsin digestion of citraconylated ovalbumin was carried out as described (5) and deblocking was carried out in 5% formic acid for 6 hr at room temperature. $NaDodSO_4$ gel electrophoresis was carried out as described (24). Silver staining was carried out as described (25).

RESULTS

Purification of I-A^d. Two types of murine Ia antigen have been described, each consisting of an α- and a β-chain. These are the I-A and the I-E complexes with the composition $A_\alpha A_\beta$ and $E_\alpha E_\beta$, respectively (26, 27). I-A^d was purified from the cell line A20-1.11, which has been shown to present antigen to a panel of I-A^d restricted T-cell hybridomas (28). Fig. 1 shows a $NaDodSO_4$/polyacrylamide gel of the purified I-A^d. The two clusters of bands with apparent M_rs of about 34,000 and 29,000 are interpreted to be the A_α and A_β chains, respectively. The heterogeneity may be due to different stages of glycosylation and post-translational processing of the two chains. The band just above A_β, at M_r = 30,000, may be the invariant chain (I_i), which is known to be associated with the cytoplasmic form of the $A_\alpha A_\beta$ complex (29). Purified I-A^k showed similar staining properties (data not

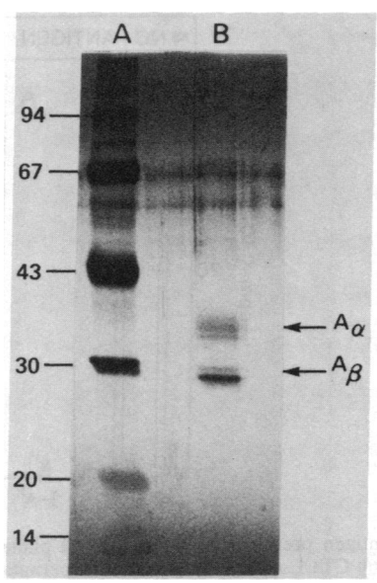

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of purified I-A^d. A 13% polyacrylamide gel run under reducing conditions and stained with silver is shown. Lane A, standard proteins with molecular weights indicated as $M_r \times 10^{-3}$ on the left; lane B, purified I-A^d, 0.3 μg.

shown). In contrast to previous purification methods (20), we did not observe any obvious contamination with actin or other cytoskeletal components. The yield of I-A^d from 10^{10} cells ranged from 474 to 660 μg, suggesting a total cellular pool of $5.7 \pm 0.9 \times 10^5$ molecules per A20-1.11 cell.

Characterization of I-A^d-Containing Planar Membranes. As has been described previously for reconstituted H-$2K^k$ (10), vesicles containing I-A^d spontaneously fuse with one another on contact with clean glass coverslips to form planar membranes. The planar membranes containing purified I-A^d bind fluorescein-labeled MKD6 (anti-I-A^d) but not fluorescein-labeled 10-2.16 antibody (anti-I-A^k). Planar membranes prepared from pure lipid vesicles also fail to bind antibody. It has been established previously (10) that when supported planar membranes are prepared from vesicles containing fluorescent lipid, the lipids diffuse normally (10^{-8} cm^2/sec) and recovery of fluorescence is 80–98% of the theoretical maximum. This shows that the supported planar membranes are continuous and not aggregated vesicles. This is also supported by the fact that fluorescent antibody bound to the I-A-containing membranes shows a uniform fluorescence over a large area.

Planar membranes could also be prepared from membrane vesicles containing unfractionated A20-1.11 cell membranes and these too showed specific binding of MKD6 antibody. In both cases, the antigen–antibody complexes were immobile, as determined by fluorescence recovery after photobleaching (10).

Antigen Presentation by I-A^d-Containing Planar Membranes. The ability of the I-A^d-containing planar membranes to stimulate the antigen-specific release of IL-2 from the T-cell hybridoma 3DO-54.8 is described in four separate experiments in Fig. 2. Dilution of the supernatants showed that the IL-2 was not saturating in this assay (data not shown). It can be seen in Fig. 2*A* that planar membranes prepared from purified, reconstituted I-A^d as well as planar membranes made from crude A20-1.11 cell membranes present a tryptic digest of citraconylated ovalbumin to the T cells. The amount of peptide antigen was not limiting (data not shown). The amount of I-A per planar membrane is determined by the phospholipid-to-protein ratio and was estimated to be 46 ng per coverslip at the maximal concentration used. No response was observed in the absence of either antigen or I-A^d or when I-A^d was replaced by I-A^k. In Fig. 2*B* it can be seen that the response was inhibited by the addition of MKD6 antibody (I-A^d-specific) but not by the addition of a comparable amount of 10-2.16 antibody (I-A^k-specific). Fig. 2 *C* and *D* show that the response to purified I-A^d was dose dependent, but the magnitude of the response to the I-A^d differed in the two experiments. It can also be seen in Fig. 2*D* that there was no response when the tryptic digest was replaced by native ovalbumin. It should also be noted that when I-A-containing vesicles were incubated on the bottom of the plastic wells in the absence of a glass coverslip, no response was observed, indicating that the surface is important in the formation of the planar membranes (data not shown).

Inhibition by GK1.5. Fig. 3 shows that the response of 3DO-54.8 to peptide and I-A^d-containing planar membranes was inhibited by the antibody GK1.5, which binds to the T-cell surface antigen L3T4. On the other hand, little or no inhibition was obtained by using the antibody I21/7.7, which recognizes another T-cell surface antigen LFA-1 even though the cell line 3DO-54.8 expresses both antigens in comparable amounts (12). In order to show that the inhibition by GK1.5 culture supernatant was not due to some nonspecific toxic effect, the supernatant was tested against the hybridoma 3DO-18.3 (5) and was found not to be inhibitory (data not shown). It has been shown previously that the I-A^d-restricted response of this hybridoma to ovalbumin is insensitive to GK1.5 inhibition (12).

Antigen Presentation by I-A^d-Containing Vesicles. Fig. 4*A* shows that vesicles prepared by dialysis of detergent-solubilized A20-1.11 membranes present antigen to 3DO-54.8 cells. However, a higher dose of membranes was required to get comparable stimulation to that obtained with glutaraldehyde-fixed A20-1.11 cells. Fig. 4*B* shows that antigen presentation by crude membrane vesicles was also inhibited by antibody. As shown in Fig. 4*C*, vesicles containing purified I-A^d were completely ineffective in antigen presentation, even though the dose of I-A^d was 10^8 cell equivalents per well.

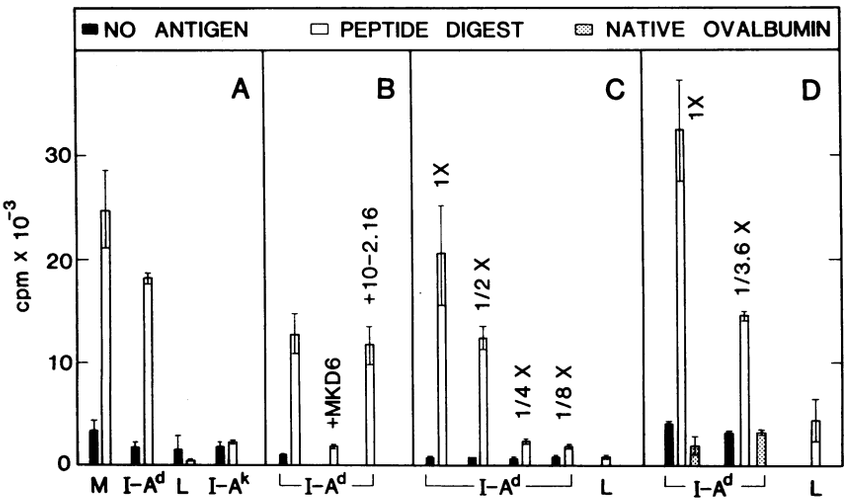

FIG. 2. Antigen presentation by supported planar membranes containing I-A^d. Results are expressed as the amount of [^{3}H]thymidine incorporated by CTLL cells in response to supernatants from 3DO-54.8 cells cultured with planar membranes. The average of four values is shown from duplicate supernatants from duplicate planar membrane experiments. Error bars represent standard error of the mean. The peptide digest was a tryptic digest of citraconylated ovalbumin (250 μg/ml, final concentration). Native ovalbumin was used at 1 mg/ml, final concentration. M represents planar membranes made from crude A20-1.11 cell membrane vesicles (3×10^4 cell equivalents per coverslip), I-A^d and I-A^k represent planar membranes containing the purified proteins at 46 ng per coverslip (10^6 cell equivalents), and L represents planar membranes made with lipid only. (*B*) Antibodies MKD6 and 10-2.16 were added to cultures to a final concentration of 6.7 μg/ml. (*C* and *D*) The I-A^d-containing planar membranes contain a constant amount of lipid and serial dilutions of I-A^d, starting at 46 ng per coverslip.

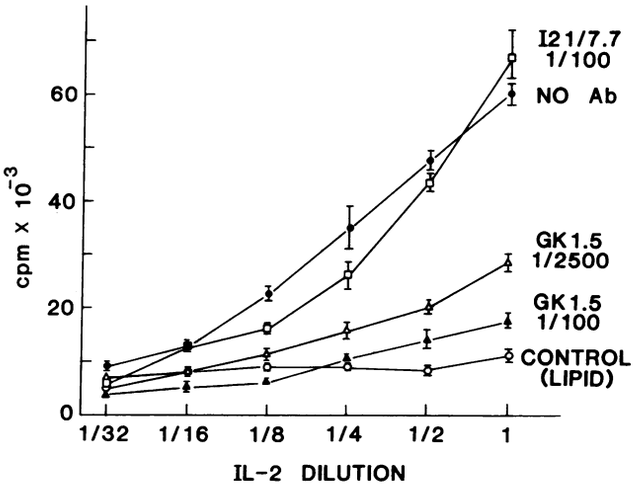

FIG. 3. Inhibition by anti-L3T4 antibody. Results are expressed as [^{3}H]thymidine incorporated by CTLL cells in response to various dilutions of IL-2-containing 3DO-54.8 culture supernatant after incubation with antigen and planar membranes containing lipid only (○) or I-A^d and lipid (●, □, △, ▲). The antibodies GK1.5 (anti-L3T4) and I21/7.7 (anti-LFA-1) were added as indicated.

DISCUSSION

The above experiments demonstrate that purified I-A^d, reconstituted into planar membranes can stimulate the antigen-specific release of IL-2 from the T-cell hybridoma 3DO-54.8. This result shows that I-A in a planar membrane together with free peptide is sufficient to stimulate this response. However, I-A^d in small unilammellar vesicles was completely ineffective in antigen presentation to these cells. There are a number of possible explanations for this difference. Vesicles prepared from sonicated lipid suspensions by the detergent dialysis procedure are small (of the order of 100 nm) and therefore tend to remain suspended in solution while the T cells settle on the bottom of the culture dish within about 10 min. On the other hand, the T cells remain in contact with the planar membranes throughout the incubation period. Indeed, our preliminary results show that IL-2 production ceases rapidly upon removal of the T cells from the planar membranes. The large surface area of the planar membranes may be important in allowing contact between the T cell and the stimulating membrane over a larger area than do the small unilamellar vesicles.

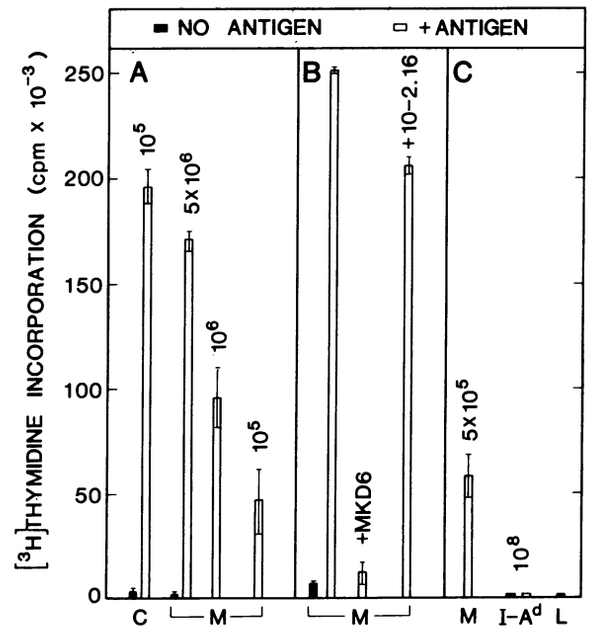

FIG. 4. Antigen presentation by I-A^d-containing vesicles. (*A*) The antigen-presenting ability of glutaraldehyde-fixed cells (C) is compared with that of A20-1.11 cell membrane vesicles (M). The amount of membrane present is indicated above each histogram in units of cell equivalents per well. (*B*) The antibody inhibition of antigen presentation by crude membrane vesicles is shown. Antibodies MKD6 and 10-2.16 were added to a final concentration of 10 μg/ml. (*C*) The ability of crude membrane vesicles (M) to present antigen is compared with that of I-A^d-containing lipid vesicles and pure lipid vesicles (L). Results are shown for 80% IL-2-containing supernatant. Dilution of these supernatants showed that the response was not saturating (data not shown).

Another difference between the planar membranes and the vesicles is that the I-A in the planar membranes is immobile, perhaps due to contact of the membranes with the glass substrate. It has been suggested that stimulation of an allogeneic cytotoxic response to H-2K^k is aided by the immobilization of this antigen by interaction with the cytoskeleton (30). It is conceivable that the planar membrane support replaces the cytoskeleton in this function. However, attempts to render I-A^d-containing vesicles active in antigen presentation by including the cytoskeleton-containing detergent-insoluble matrix (30) during the reconstitution were unsuccessful. Furthermore, removal of this fraction from the crude A20-1.11 membrane preparation did not render these vesicles inactive (data not shown). In contrast, A20-1.11 cell membrane vesicles can present antigen in both the vesicular and planar forms. However, the crude membrane preparation forms large irregular-shaped aggregates, which are visible by light microscopy and are therefore much larger than the vesicles prepared from purified I-A and lipid.

Fig. 2 shows that planar membranes prepared from purified I-A^d are comparable in antigen-presenting ability to planar membranes prepared from A20-1.11 cell membranes, even though the crude membranes have 1/30th as much I-A^d. There are several possible explanations for this difference. The elution of the I-A from the affinity column may have resulted in some irreversible denaturation. Also, the fact that the I-A^d was more completely solubilized prior to reconstitution may result in less of the I-A^d being inserted in the correct orientation. Another possibility is that the planar membranes prepared from purified I-A lack additional molecules that stabilize the interaction between the T cell and the antigen-presenting membrane.

The data in Fig. 3 show that the response to peptide antigen and I-A^d was inhibited by anti-L3T4 antibody but not by anti-LFA-1 antibody, even though both antigens are present in comparable amounts on the T-cell surface (12). This suggests that the L3T4 antigen is involved in the recognition/activation process. One must be cautious in interpreting this result as indicating that L3T4 binds directly to Ia (12, 13), since it is also possible that it binds to phospholipid or that L3T4 is involved in T-cell surface events that occur subsequent to I-A/peptide recognition.

Despite the fact that the response to purified I-A^d in planar membranes is somewhat smaller than the response to antigen presented by fixed cells, the signal is sufficiently large (5–30 times background) that it offers an attractive system for studying the details of T-cell receptor/Ia/peptide antigen interaction in a greatly simplified model system. Recently, the active peptide fragment responsible for the stimulation of IL-2 release by 3DO-54.8 cells has been identified (8). This should allow us to use purified fluorescent peptide as well as purified fluorescent I-A^d to study the interaction between these molecules before and during T-cell binding. Furthermore, the fact that I-A^d-containing planar membranes can replace the antigen-presenting cell, when the antigen is provided in a processed form, should be useful in delineating the

role of lymphokines in the generation of T-cell help. In addition, the use of planar membranes instead of fixed cells in determining the form of antigen that is ultimately recognized by the T cell offers the advantage that it removes doubts as to whether processing has been completely inhibited by the mild fixing procedures used.

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