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CHARACTERIZATION OF AN ANTI-H-2 MONOCLONAL ANTIBODY AND ITS USE IN LARGE-SCALE ANTIGEN PURIFICATION[1]

KATHRYN C. STALLCUP,[2] TIMOTHY A. SPRINGER, AND MATTHEW F. MESCHER

From the Department of Pathology, Harvard Medical School, Boston MA 02115

A rat anti-mouse monoclonal antibody (MAb), M1/42, has been found to react with H-2 antigens from cells of the a, b, d, j, k, s, and u haplotypes (all haplotypes tested). This antibody, when bound to cells and reacted with FITC-conjugated anti-rat Ig, could be used to quantitate H-2 expression on several cell types. The antibody was also useful in comparing the H-2 products precipitated from a variety of haplotypes. M1/42-coupled Sepharose-4B beads were used to purify H-2^d antigens by affinity chromatography. Pure H-2 molecules eluted from the column in 0.5% DOC, 0.65 M NaCl, 20 mM Tris, pH 8.0, yielding 110 to 180 μg H-2^d/10^{10} P815 tumor cells. This antibody, when used in series with H-2K^k-specific MAb 11-4.1, allowed purification of D^k and D^d from RDM-4 and YAC cells, respectively. H-2^d purified by column chromatography on M1/42 was found to be serologically and biologically active, as determined by MAb rebinding, inhibition of cell lysis by alloantisera plus complement, and ability to stimulate alloreactive CTL. This antibody and the described protocols should be useful in the preparation of relatively large quantities of a number of H-2 antigens.

Cell surface antigens encoded by the major histocompatibility complex (MHC)[3] play important roles in many immune system phenomena. The glycoproteins coded for by the murine H-2K, H-2D, and H-2L genes are involved in allograft rejection and are recognized by the cytotoxic T lymphocytes (CTL) that lyse allogeneic, virus-infected, chemically modified, or transformed cells (1, 2). The polymorphism of the H-2 antigens and their importance in various immune responses have led to a great deal of interest in the structure and function of these molecules.

Murine H-2K, -D, and -L antigens, and the analogous human HLA-A, -B, and -C antigens, are 2-chain structures formed by noncovalent interactions between a heavy chain (m.w. approximately 44–50,000: the MHC gene product), and β_2-microglobulin (m.w. approximately 13,000) (3, 4). The heavy chain spans the membrane bilayer and is glycosylated (3–5). The primary structure of the polymorphic heavy chains has been studied using a number of methods. In the case of HLA-A, -B, and -C, conventional methods of protein biochemistry (4, 6) have been used to study the relatively large quantities of antigen that can be purified from Epstein-Barr virus-transformed human cell lines (7).

Large quantities of purified H-2 antigens have not previously been available, and most studies of their structure have been done using small amounts of radioactively labeled protein (8, 9). Although much structural information has been gained by this approach, further structural, and particularly functional, studies would be facilitated by the availability of large amounts of pure H-2. There have been several reports of purification of murine MHC antigens by conventional methods, but the protocols are lengthy and give relatively low yields (10–12). Herrmann and Mescher (13) have described a rapid, efficient procedure for affinity column purification of the H-2K^k antigen using a monoclonal antibody (MAb) specific for H-2K^k (14). A similar procedure for purification of HLA antigens using MAb has been described by Parham (15).

This report describes a new monoclonal antibody, M1/42, which binds to the H-2 antigens of a variety of haplotypes. No allogeneic specificity has been detected in the strains tested. The MAb binds

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[3] Abbreviations used in this paper: MAb, monoclonal antibody; CTL, cytotoxic T lymphocyte(s); Triton/Tris buffer, 0.5% Triton X100, 20 mM Tris, pH 8.0; PMSF, phenylmethylsulfonylfluoride; DOC, deoxycholate; NP40, Nonidet P-40; DOC/0.65 M NaCl buffer, 0.5% DOC, 20 mM Tris, pH 8, 0.65 M NaCl; PBES, phosphate-buffered Earle's balanced salts solution; LPO, lactoperoxidase; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; MHC, major histocompatibility complex; OVA, ovalbumin; Ribo A, ribonuclease A; M_r, relative molecular mass.

tightly enough to allow its use for affinity column purification of H-2, yet the antigens can be eluted under mild conditions. Using this antibody, we have been able to rapidly isolate relatively large quantities of serologically and biologically active H-2K and D antigens from several haplotypes. These results, together with those previously reported (14–16), demonstrate that MAb against MHC antigens can be useful as affinity purification reagents.

MATERIALS AND METHODS

Mice and tumor cell lines. All mice were purchased from The Jackson Laboratory (Bar Harbor, ME) with the exceptions noted below. YAC lymphoma ($H\text{-}2^a$), EL-4 lymphoma ($H\text{-}2^b$), RDM-4 ($H\text{-}2^k$) lymphoma, and P815 mastocytoma ($H\text{-}2^d$) cells were maintained as ascites in C57BL/6, (C57BL/6 × A/J)F_1 (B6 × A), AKR, and (BALB/c × DBA/2)F_1 ($CD2F_1$) (Cumberland View Farms, Clinton, TN) mice, respectively. I/st ($H\text{-}2^i$) mice and I.29 lymphoma cells were generous gifts from Dr. Nobuhiko Tada, Memorial Sloan-Kettering Cancer Center, New York, NY.

M1/42 monoclonal antibody. The derivation of M1/42 MAb has previously been described (17). It was 1 of 12 rat anti-mouse spleen cell MAb obtained after immunization of a DA rat to B10 spleen cells and fusion with the mouse myeloma line NSI. The hybridoma line has been stabilized by 2 reclonings (M1/42.3.9.8), and secretes an IgG2a HLK antibody (containing specific H and L and myeloma K chains) into tissue culture medium to a concentration of about 100 μg/ml. This hybridoma cell line is available from the Salk Institute Cell Distribution Center, North Torrey Pines Road, La Jolla, CA.

M1/42 antibody was purified from culture supernatant by ammonium sulfate precipitation and Sephadex G-200 (Pharmacia, Piscataway, NJ) chromatography. The purified antibody was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) at 2.5 to 3 mg/ml of wet gel.

Surface labeling with ^{125}I. Lactoperoxidase-catalyzed iodination was used to label cell surface proteins. Iodination was done at 25°C using 0.2 mCi of carrier-free ^{125}I (New England Nuclear, Boston, MA) and 10 μg of lactoperoxidase (Calbiochem, San Diego, CA) per 5×10^6 cells. The labeling procedure was as described (18) except that the final 2 post-iodination washes were done in phosphate-buffered Earle's balanced salts solution (PBES, GIBCO, Grand Island, NY) or medium RPMI 1640 (GIBCO) medium containing 10% fetal calf serum. Incorporation of label was 1 to 4 cpm/cell.

Immunoprecipitation of H-2 antigens. Iodinated cells were lysed in 0.5% Triton X-100, 20 mM Tris, pH 8.0 (Triton/Tris buffer) containing 1 to 2 mM phenylmethylsulfonylfluoride (PMSF) at 5×10^7 cells/ml. Nuclei were removed by centrifugaton, and M1/42-Sepharose beads were added to the supernatant. Incubation was for 1 hr at 4°C with shaking. The beads were then washed 4 times with 0.5 ml Triton/Tris buffer. Precipitation with 11-4.1 was as described above, except that after 1 hr the soluble antigen-antibody complexes were precipitated by the addition of protein-A-Sepharose beads (Pharmacia) followed by incubation (with shaking) for another hour. Material bound to the antibodies was eluted with the sample buffer described by Laemmli (19).

Detergent solubilization of antigen. When whole cell lysates were used, cells were lysed at 2×10^7 cells/ml in Triton/Tris buffer containing 1 to 2 mM PMSF (all reagents from Sigma Chemical Co., St. Louis, MO). After 10 min on ice, nuclei were pelleted at 3600 × G for 10 min and the resulting supernatant was cleared of detergent insoluble material by centrifugation for 45 min at 100,000 × G. The resulting high speed supernatant was loaded onto affinity columns.

When necessary, cell fractionation was done using the protocol described by Lemonnier *et al.* (20), and is shown schematically in Figure 3. H-2 antigens could be recovered from both the low-speed pellet and high-speed pellet (see Fig. 3) by solubilization with nonionic detergent. The low-speed pellet, consisting of nuclei and membrane fragments, was solubilized in Triton/Tris buffer containing 1 to 2 mM PMSF for 10 min on ice. Nuclei and detergent insoluble material were pelleted as above, and the resulting high speed supernatant was loaded on to affinity columns. The crude membrane pellet (see Fig. 3) was solubilized in 1.0% Triton X-100, 20 mM Tris, pH 8.0, 1 to 2 mM PMSF at a detergent:protein ratio of 5:1. Detergent-insoluble material was pelleted as above, and the supernatant was loaded onto affinity columns.

Affinity chromatography. Detergent lysate or detergent solubilized proteins (containing a small amount of ^{125}I-labeled surface protein) were passed through a column of M1/42-Sepharose (0.5 ml to 0.9 ml). In most cases, the starting material was first passed through a pre-column of Sepharose 4B (1 ml). The M1/42 column was then extensively washed with Triton/Tris buffer, usually 25 to 30 column volumes, until ^{125}I radioactivity reached background levels. Elution of bound antigen was done using 0.5% deoxycholate (DOC) in 20 mM Tris, pH 8, with 0.65 M NaCl (DOC/0.65 M NaCl buffer) unless otherwise indicated. Fractions of about 0.9 ml were collected, and the ^{125}I radioactivity was measured. Peak fractions were pooled, samples were taken for protein determination by the method of Lowry *et al.* (21), and the remaining H-2 antigens were frozen until further use. Samples containing 10 to 20 μg of protein were precipitated with 6 vol of cold acetone for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Separation of H-2 heavy chain and β_2-microglobulin. ^{125}I-labeled $H\text{-}2K^k$ was purified by affinity chromatography on MAb 11-4.1 (13). The sample (containing 0.3 to 1.0×10^7 cpm of ^{125}I) was precipitated with 6 vol of cold acetone, resuspended in approximately 2 ml 6 M guanidine, and incubated for 20 to 30 min at room temperature. Heavy chain and β_2-microglobulin were then separated by chromatography on a 1 x 120 cm Biogel A 0.5M (BioRad, Richmond, CA) column in 6 M guanidine, 20 mM Tris, pH 8. Fractions containing peaks of radioactivity were pooled, and samples containing either β_2-microglobulin, heavy chain, or a mixture of heavy chain and β_2-microglobulin (2:1 heavy:light) were dialyzed extensively against 20 mM Tris, pH 8.0, to remove guanidine. After dialysis, Nonidet P-40 (NP40; Particle Data Labs, Evansville, IL) was added to a final concentration of 0.5% and the samples were loaded directly onto M1/42 columns to determine binding.

CTL stimulation using H-2-containing liposomes. Liposomes containing purified $H\text{-}2K^k$ or $H\text{-}2^d$ proteins were prepared as described (22). Briefly, 1 μg $H\text{-}2^d$ in DOC/0.65 M NaCl buffer was mixed with 4.6 nmol lipid (chloroform:methanol extracted from P815 cells) and 2.8×10^7 cell equivalents of detergent insoluble matrix (from P815 cells) (23). Detergent was removed by dialysis against Tris-buffered saline, pH 8. Responder spleen cells were from $CD2F_1$ mice previously primed with YAC cells or C57BL/6 mice primed with P815 cells. CTL response was measured 4 to 5 days later using a standard ^{51}Cr release assay with P815 ($H\text{-}2^d$) or RDM-4 ($H\text{-}2^k$) tumor cell targets.

Cytotoxicity assay. M1/42-column purified $H\text{-}2^d$ antigens were dialyzed against PBS to remove detergent. Allogeneic activity was assayed by adding 2-fold dilutions of pure antigens to a standard cytotoxicity-inhibition assay using ^{51}Cr-labeled spleen cell targets and conventional alloantisera.

SDS-PAGE. Electrophoresis, in the presence of SDS and β-mercaptoethanol, was done by the method of Laemmli (19). The running gel consisted of a gradient of 5 to 15% polyacrylamide (BioRad). Gels were stained with Coomassie Blue, and autoradiography of dried gels was done using Kodak XR-5 x-ray film.

RESULTS

Cell binding of M1/42. The rat MAb M1/42 was raised by immunizing rats with spleen cells from B10 ($H\text{-}2^b$) mice. Screening of M1/42 reactivity by cell-binding assay (Table I) indicated that the antibody had no identifiable strain or haplotype specificity. In

TABLE I

Specificity screening of MAb M1/42

Haplotype	Strain/Cell Type		Binding[a]	MHC Precip.[b]
a	B10.A	s.c.[c]	+	ND
	YAC	lymphoma	ND	+
b	C57/LJ	s.c.	+	ND
	C57BL/10	s.c.	+	ND
	C57BL/6	s.c.	+ (FACS)	+
	B10	s.c.	+	ND
	EL-4	lymphoma	+ (FACS)	+
d	LG/J	s.c.	+	ND
	B10.D2	s.c.	+ (FACS)	ND
	$CD2F_1$	s.c.	ND	+
	P815	mastocytoma	+ (FACS)	+
h2	B10.A (2R)	s.c.	+	ND
i5	B10.A (5R)	s.c.	+	ND
j	I/St	s.c.	ND	+
	I.29	lymphoma	ND	+
k	B10.Br	s.c.	ND	+
	AKR	s.c.	ND	+
	RDM-4	lymphoma	+ (FACS)	+
s	B10.S	s.c.	ND	+
u	PL/J	s.c.	+	ND

[a] Binding was measured by using M1/42 MAb followed by either ^{125}I- or FITC-labeled rabbit $F(ab')_2$ anti-rat IgG (27). Radioactive binding or fluorescent intensity of ×2 or more over background was taken as positive.

[b] A positive indicates that M1/42-Sepharose precipitated polypeptides with m.w. of 44,000 to 50,000 and 13,000 from ^{125}I-labeled cell lysates.

[c] s.c., spleen cells.

addition, M1/42 did not distinguish normal spleen cells from transformed cells.

Immunoprecipitation of ^{125}I-labeled cell surface antigens with M1/42. Cell-binding studies demonstrated that M1/42 could interact with molecules on the cell surface of all mouse haplotypes tested. The surface antigens recognized by the MAb were visualized by radioactive labeling followed by immunoprecipitation. AKR (H-2^k) spleen cells were surface labeled using lactoperoxidase-(LPO) catalyzed iodination and lysed in 0.5% NP40, 20 mM Tris, pH 8 (containing PMSF). The lysate was incubated with either M1/42-coupled Sepharose 4B beads or MAb 11-4.1 (αK^k) followed by *Staphylococcus aureus* protein A-Sepharose. M1/42 and 11-4.1 both precipitated proteins with relative molecular mass (M_r) of ~47,000 and 12,000 from H-2^k spleen cells (Fig. 1), suggesting that M1/42 was recognizing H-2 antigens. Reactivity of M1/42 with H-2 was shown by preclearing experiments. AKR spleen cell lysates were treated with either M1/42-Sepharose or 11-4.1-protein A-Sepharose, and the remaining supernatants were tested for residual reactivity with either antibody. Precipitated molecules were eluted from the beads and subjected to SDS-PAGE. Treatment of lysates with M1/42 removed all K^k molecules that are recognized by 11-4.1 (Fig. 1*e*), while treatment of lysates with 11-4.1 removed all K^k (Fig. 1*a* and *b*) but not all molecules reactive with M1/42 (Fig. 1*c*). The electrophoretic mobility of the remaining M1/42-reactive molecules is typical for H-2 antigens, indicating that M1/42 probably recognizes D^k as well as K^k. This data and other results shown below demonstrate that M1/42 reacts with H-2 antigens of many haplotypes and appears to react equally well with both H-2K-end and H-2D-end products of d and k haplotypes.

Precipitation of antigens from several other haplotypes confirm these conclusions (Fig. 2), since in all cases M1/42 reacted with heavy chain(s) in the 45–50,000-dalton region and a light chain that migrates in the position of β_2-microglobulin. In several cases the heavy chains show differences in m.w. For instance, treatment of P815 (H-2^d) lysates with M1/42 resulted in precipitation of 2 distinct heavy chains (Fig. 2*d*). These 2 bands probably represent the K^d and D^d proteins, with the difference in m.w. consistent with that reported by Krakauer *et al.* (24). The identification of the lower band as D^d is further supported by precipitation of MHC products from YAC cells (H-2^a: K^d, D^d) (Fig. 2). Only 1 band is precipitated when YAC cell lysates are treated with M1/42 (Fig. 2*c*). This band appears to represent both the K^k and D^d proteins, as predicted by their similar m.w. (24), and directly shown by preclearing the lysate using the K^k-specific MAb 11-4.1 followed by precipitation of D^d with M1/42 (Fig. 2*b*).

M1/42 precipitates a single wide band from C57BL/6 spleen cells (H-2^b) (data not shown). It has been reported that M1/42

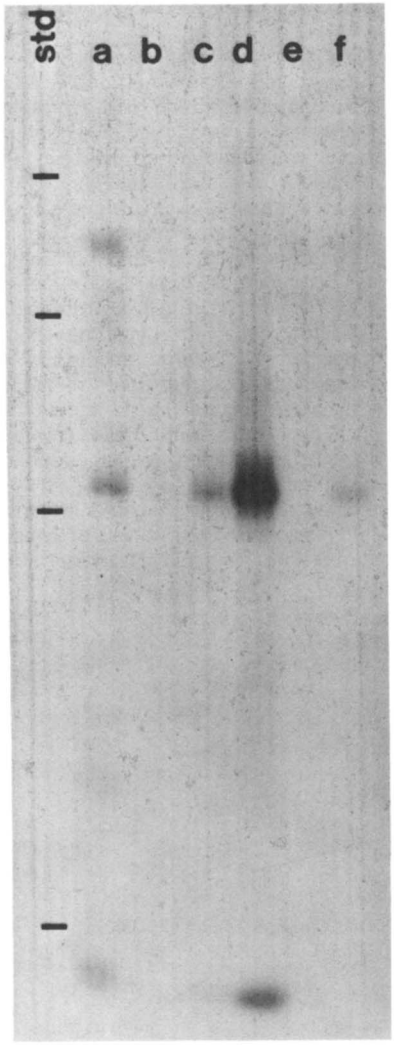

Figure 1. **Sequential precipitation of H-2^k antigens using M1/42 and 11-4.1.** Spleen cells from AKR (H-2^k) mice were iodinated as described in *Materials and Methods*. Surface-labeled cells were lysed in Triton/Tris buffer containing PMSF, nuclei were removed, and 300 λ of the supernatant was allowed to react for 1 hr with either 200 λ of M1/42-Sepharose beads or 75 λ of 11-4.1 (at 1 mg/ml) followed by protein A-Sepharose. At the end of the reaction time the beads were pelleted, each supernatant was divided into equal aliquots and the precipitations with M1/42-Sepharose or 11-4.1 and protein A-Sepharose were repeated. Material bound to beads was eluted with SDS gel sample buffer and subjected to electrophoresis and autoradiography as described in *Materials and Methods*. Standards are phosphorylase A (M_r = 94,000), bovine serum albumin (BSA) (M_r = 68,000), ovalbumin (OVA) (M_r = 45,000), and ribonuclease A (Ribo A) (M_r = 13,700). *a*, precipitation with MAb 11-4.1; *b*, supernatant from (*a*) followed by precipitation with 11-4.1; *c*, supernatant from (*a*) followed by precipitation with MAb M1/42; *d*, precipitation with MAb M1/42; *e*, supernatant from (*d*) followed by precipitation with MAb 11-4.1; *f*, supernatant from (*d*) followed by precipitation with MAb M1/42.

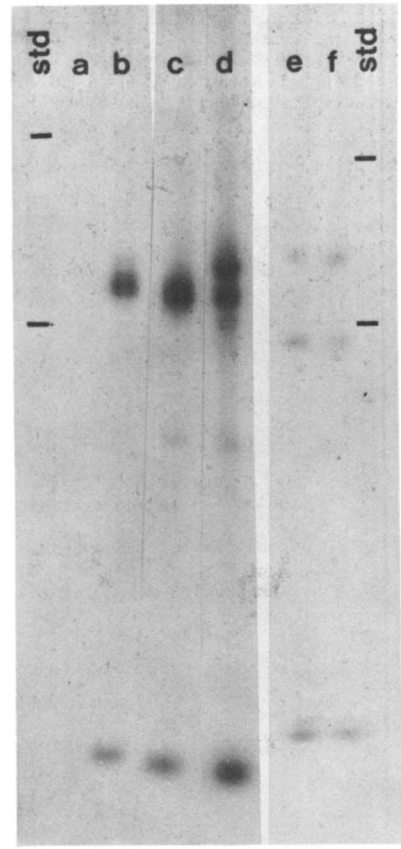

Figure 2. **MAb M1/42 precipitation of H-2 antigens from cells of the a, d, and j haplotypes. Cells were ^{125}I-labeled as described in *Materials and Methods*** and precipitations done as described in the legend to Figure 1. *a*, precipitation by 11-4.1 from YAC (H-2K^{k}D^d) lysate precleared with 11-4.1; *b*, precipitation by M1/42 from YAC lysate precleared with 11-4.1; *c*, precipitation by M1/42 from YAC lysate without preclearing; *d*, precipitation by M1/42 from P815 (H-2K^{d}D^d) lysate; *e*, precipitation by M1/42 from H-2^j spleen cell lysates; *f*, precipitation by M1/42 from I.29 (H-2^j) lymphoma lysate.

recognizes only the H-2K end of the b haplotype (17). A particularly interesting pattern was found after precipitation of spleen or lymphoma cells of the H-2^{i} ($K^{i}D^{b}$) haplotype (Fig. 2*e* and *f*). Both spleen and lymphoma cells yielded 2 heavy-chain bands, 1 fairly large (M_r = ~48,000) and 1 quite small (M_r = ~38,000). It is not clear whether the smaller band represents an intact H-2 molecule or a breakdown product, although H-2 protein breakdown is usually not observed under the conditions of these immunoprecipitations.

Quantitation of M1/42 binding to various cell types. The specificity of M1/42 for H-2 antigens and the apparent lack of alloantigenic specificity suggested that M1/42 might provide a means of purifying a variety of H-2 antigens. We first examined several possible sources with respect to the quantity of H-2 expressed on the cell surface, since purification can be greatly facilitated by the use of cells that produce large amounts of the antigen. Herrmann and Mescher (13) had noted that H-2K^{k} is expressed in large amounts on RDM-4 cells, comparable to the expression of HLA antigens on Epstein-Barr virus-transformed human cells (7).

Cells were stained with M1/42 MAb and mouse IgG-absorbed fluorescein isothiocyanate- (FITC) coupled rabbit F(ab')$_2$ anti-rat IgG under saturating or near-saturating conditions and analyzed on the fluorescence-activated cell sorter (FACS) (Table II). Fluorescent intensities thus obtained were directly proportional to the number of antibodies bound per cell. T cell and B cell blasts and YAC, P815, EL-4, and P388D$_1$ cell lines expressed 2.3- to 3.5-fold more H-2 than normal spleen cells. Among the lines studied here, RDM-4 expressed unusually high amounts of H-2, approximately 10-fold more than spleen cells, confirming previous results (13). Light-scattering measurements on the FACS and measurement of cell size under the microscope suggested that RDM-4 was smaller or similar in size to the other tumor cells, indicating that the increased expression of H-2 on this line is not simply due to greater surface area. The RIE/TL8X.1 mutant line, previously characterized as negative for Tla and H-2 antigens, due to a complementable mutation suggested to involve carbohydrate or β_2-microglobulin (25), expresses no material cross-reactive with M1/42 (Table II).

TABLE II

Quantitation of H-2 Expression Using M1/42 and FACS

Cell	H-2 (Spleen Cell Relative Units)[a]	Scatter Intensity (Mean Channel No.)
Spleen	(1)	52
Con A blasts[b]	2.3	134
LPS blasts[b]	3.1	155
RIE/TL8X.1	0	106
RDM-4	9.8–12.7[c]	97
YAC	3.2	106
P815	3.5	140
P388D$_1$	2.7	127
EL-4	3.5	108

[a] Cells were labeled with saturating M1/42 MAb or irrelevant MAb, then FITC-anti-rat IgG and analyzed on the FACS as described (27). The mean fluorescence intensity of each cell type was calculated by integration, corrected for background labeling, and expressed relative to the intensities of C57BL/6J splenic nucleated cells.

[b] Scatter gated to include only blasts.

[c] Two-fold more FITC anti-rat IgG gave 1.3-fold brighter labeling.

These results indicated that tumor cell lines would be a good source of H-2 for purification and suggest that M1/42 could be useful in more extensive studies of H-2 expression on various cell lines.

Purification of H-2 antigens using M1/42. Previous work in this laboratory (13) has shown that an MAb coupled to Sepharose 4B could be used for affinity purification of H-2 proteins. Since M1/42 bound H-2 antigens tightly enough to give good immunoprecipitates, it appeared that it might be an effective affinity reagent. Small-scale experiments using whole cell lysates from 5 to 25 × 10^8 cells demonstrated that H-2$^{b,d,\text{and }k}$ products could bind and be eluted from M1/42 columns (data not shown). Substantial amounts of H-2 could be eluted using 0.5% DOC in 20 mM Tris, whereas 0.5% DOC in Tris containing 0.65 M NaCl appeared to elute all material bound to the column. Using this protocol, H-2^{b} could be purified from EL-4 tumor cells with a yield of ~250 μg H-2 per 1 × 10^{10} cells. Attempts to purify large amounts of H-2 (e.g., from 10^{10} cells) using whole cell detergent lysate as a source of starting material were not always successful, particularly in the case of P815 (H-2^{d}) cells. The lysates tended to become quite viscous over the course of column loading (which, depending upon the lysate volume, could often be 36 to 50 hr at 4°C), frequently plugging the columns and generally reducing the flow rate to impractical levels. This problem was solved by separating the bulk of the cellular membranes from the cytoplasm with the scheme

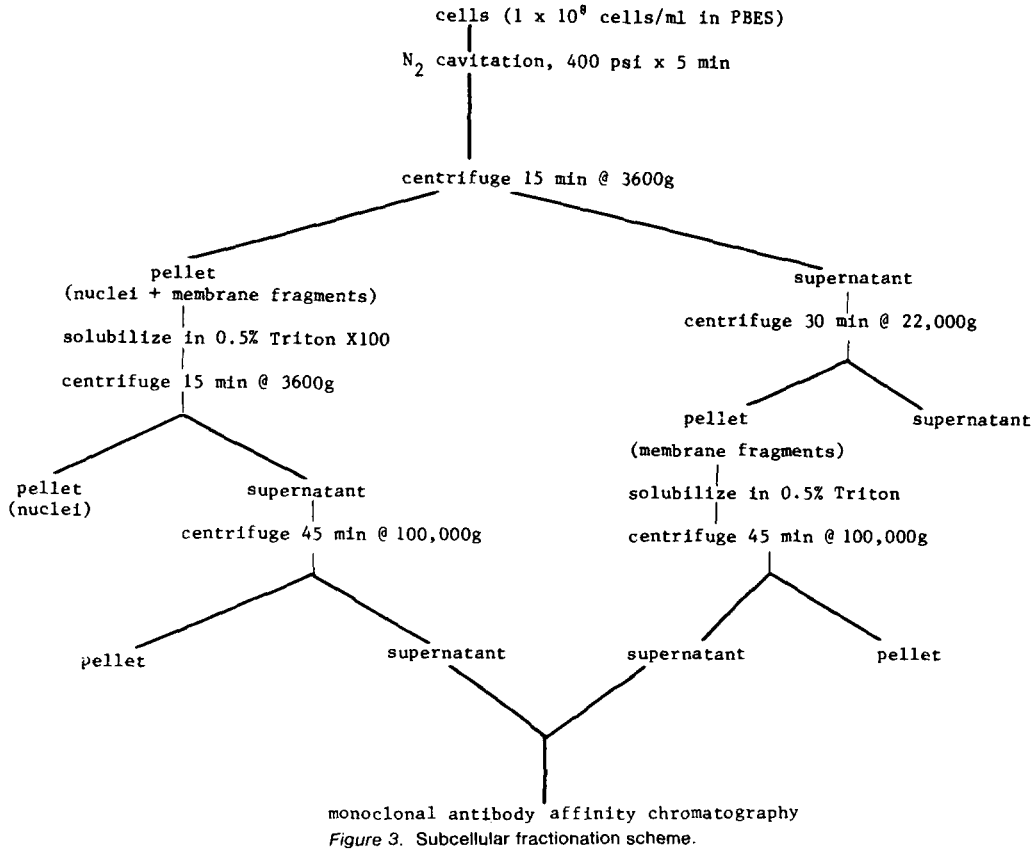

Figure 3. Subcellular fractionation scheme.

outlined in Figure 3. Nitrogen cavitation was used to lyse the cells, and membrane fragments were isolated from nuclei and cytoplasm by differential centrifugation. Previous studies (20) had shown that significant amounts of plasma membrane (assayed by 5′-nucleotidase activity) pellet with the nuclei after the 1st low-speed centrifugation. We were able to significantly increase recovery of H-2 (often by 100%) by solubilizing the low-speed pellet as well as the 22,000 × G membrane pellet. The low-speed pellet and membrane pellet were solubilized, loaded on MAb-Sepharose columns, and eluted as described in *Materials and Methods*.

The elution profile and protein composition of a typical H-2^d preparation are shown in Figure 4. Elution was monitored using trace amounts of whole cell lysate from cells surface labeled with ^{125}I. This method predictably yields between 110 and 180 μg H-2/10^{10} P815 cells. The Coomassie Blue-stained gel shown in Figure 4 demonstrates that heavy chains of both K^d and D^d and β_2-microglobulin are bound to the M1/42 column and that the eluate is free of any major contaminants. Autoradiography of the gel revealed no radioactive contaminants (data not shown). The ratio of heavy chains to β_2-microglobulin is approximately 2:1 based on ^{125}I radioactivity. Individual M1/42 columns have been used 5 to 10 times with no apparent loss in capacity.

During early, relatively small-scale preparations, the efficacy of several elution buffers was tested. Figure 5 shows the profile of a P815 whole cell lysate eluted with buffers of different detergent or salt concentrations. Gels of proteins found in the eluted samples indicate that both K^d and D^d are present in each peak (data not shown). The 1st peak showed an enrichment of H-$2K^d$; however, attempts to achieve clean separation of K^d and D^d using DOC or salt gradient elutions were unsuccessful. The heterogeneous elution is particularly curious in light of the monoclonality of M1/42 and may reflect slight heterogeneities in parts of the H-2 proteins proximal to the antigenic determinant. Alternatively, heterogeneous elution may reflect heterogeneity of the antibody produced by the M1/42 clone, since the rat Ig heavy chain could combine with either a specific rat Ig light chain or the myeloma κ-chain. Standard large-scale preparations are eluted with DOC/0.65 M NaCl buffer, a buffer that elutes all of the bound antigen.

Using M1/42 as 1 of a series of columns, it becomes possible to isolate individual K or D antigens. Figures 6 and 7 show the purification of H-$2D^d$ from YAC cells after using MAb 11-4.1 to isolate K^k. The flow-through from the αK^k column, when completely cleared of K^k, was allowed to bind to M1/42, and D^d was subsequently eluted. We have used a similar protocol to isolate D^k from RDM-4 cells (H-2^k). In the case of RDM-4 cells, the material that elutes from M1/42 represents approximately 10 to 14% of the material identified as K^k. This is in agreement with the fact that RDM-4 cells over-express H-$2K^k$. The possibility exists that M1/42 does not bind H-$2D^k$ and that the material bound is partially denatured H-$2K^k$ that did not bind to the 11-4.1 column. This is made unlikely by the results of the preclearing experiment shown in Figure 1 and the results described below.

M1/42 does not bind separated heavy and β_2-microglobulin chains. The fact that M1/42 bound to a large number of H-2 molecules suggested that the MAb could be directed toward either a determinant on β_2-microglobulin or a region common to all heavy chains, a region that might retain antigenicity even after partial denaturation. To test this possibility, we isolated ^{125}I-labeled H-$2K^k$ by affinity chromatography on MAb 11-4.1. The heavy and light chains were dissociated in 6 M guanidine HCl and separated by chromatography on Biogel A 0.5M. The separated chains were dialyzed into 0.5% Triton X-100, 20 mM Tris, or alternatively, separated heavy chain and β_2-microglobulin were mixed (2:1 heavy chain:β_2-microglobulin) and dialyzed together. Samples were applied to M1/42, and the counts bound *vs* counts in the flow-through were determined. Table III shows that 64% of the pure, native K^k bound to M1/42, whereas a mixture of K^k and β_2-microglobulin did not bind at all. Separated β_2-microglobulin and heavy chains each bound poorly, if at all. Although these data do not rule out the possibility that M1/42 recognizes β_2-microglobulin or partially denatured heavy chain, it seems less likely. These data also argue against the likelihood of M1/42 binding denatured H-$2K^k$ instead of H-$2D^k$ in MAb 11-4.1-absorbed RDM-4 lysates.

Serologic and biologic activity of H-2 antigens purified on M1/42 columns. Affinity chromatography using MAb 11-4.1 columns results in excellent recovery of H-$2K^k$ serologic and biologic activity (13, 22). Similarly mild elution conditions for M1/42 suggested that H-2 antigens purified with this MAb should also retain activity. This was confirmed using both serologic and biologic criteria.

Serologic activity was tested by rebinding purified H-2^b and H-

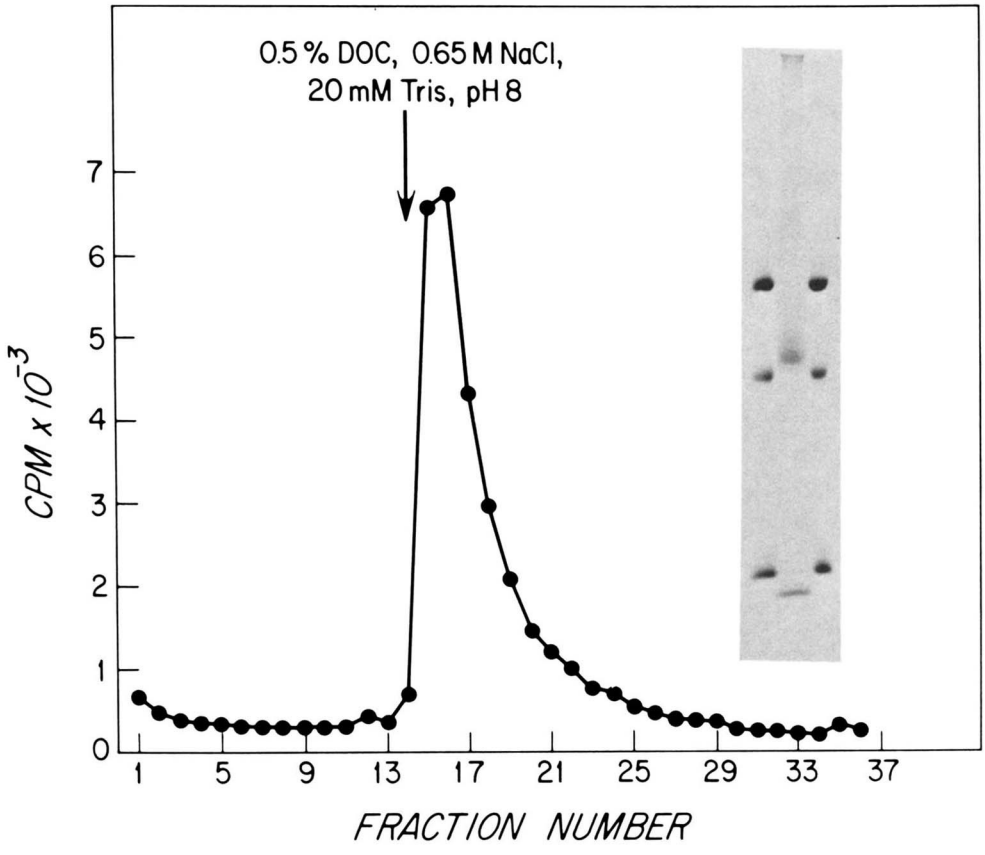

Figure 4. Elution profile and SDS-PAGE of H-2^d antigens purified by affinity chromatography on M1/42. The high speed pellet (including ^{125}I-labeled surface proteins as tracer) (see Fig. 3) from P815 cells was solubilized with Triton X-100 and loaded onto M1/42 as described in the text. The column was washed extensively with Triton/Tris buffer and fractions (numbers 1–13) were collected at the end of the wash. DOC/0.65 M NaCl elution buffer was then added and elution was monitored by following the ^{125}I tracer. Fractions 15–18 were pooled and 10 to 20 μg of protein was precipitated with 6 vol cold acetone and analyzed by SDS gel electrophoresis (*inset*). The gel was stained with Coomassie Blue and the markers in the outside lanes are BSA, OVA, and Ribo A. Similar results were obtained when H-2 was purified from the low speed pellet (nuclei and membrane fragments, see Fig. 3) from these P815 cells.

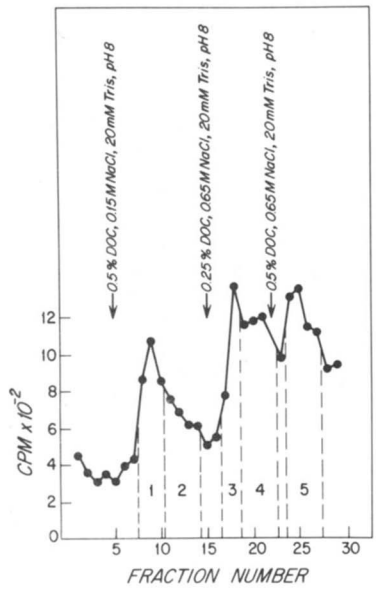

Figure 5. H-2^d antigens elute heterogeneously under some conditions. Whole P815 cells were lysed with Triton X-100 and loaded onto an M1/42 column as described in the text. After extensive washing, a stepwise elution was done using the indicated buffers. Three to 4 peaks of radioactivity were resolved. Fractions across the elution profile were combined into 5 pools, as shown.

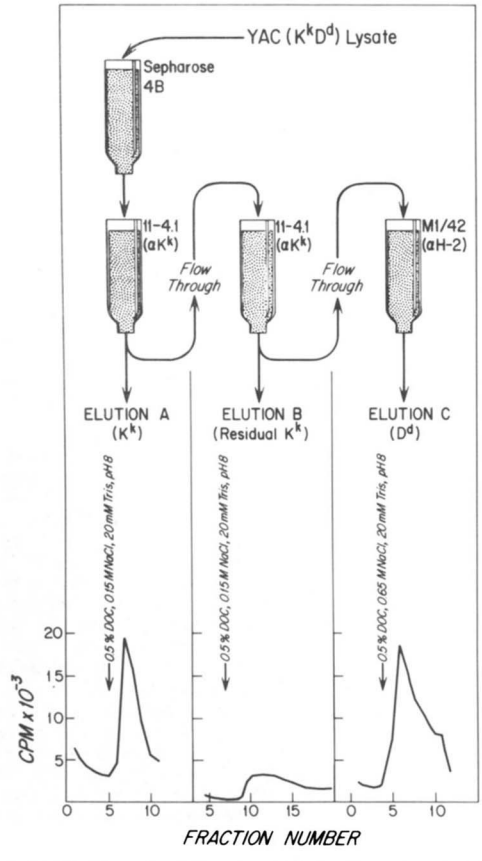

Figure 6. Purification of H-2K^k and H-2D^d. Seven and one-half × 10^7 YAC cells were iodinated, lysed in Triton/Tris buffer, loaded through a 0.75 ml Sepharose 4B precolumn and onto a 0.5 ml MAb 11-4.1 column. The flow-through was collected and saved. The 11-4.1 column was washed with Triton/Tris buffer, then eluted with 0.5% DOC/TBS and collected as 60 drop fractions (*elution a*). The 11-4.1 column was re-equilibrated with Triton/Tris and the flow-through from the 1st column application was loaded in series through the 11-4.1 column and a 0.8 ml column of M1/42-Sepharose. Residual K^k was eluted from 11-4.1 as above (*elution B*). The M1/42 column was washed with 15 to 20 vol of Triton/Tris, then eluted with 0.5% DOC/0.65 M NaCl buffer into 60 drop fractions (*elution C*).

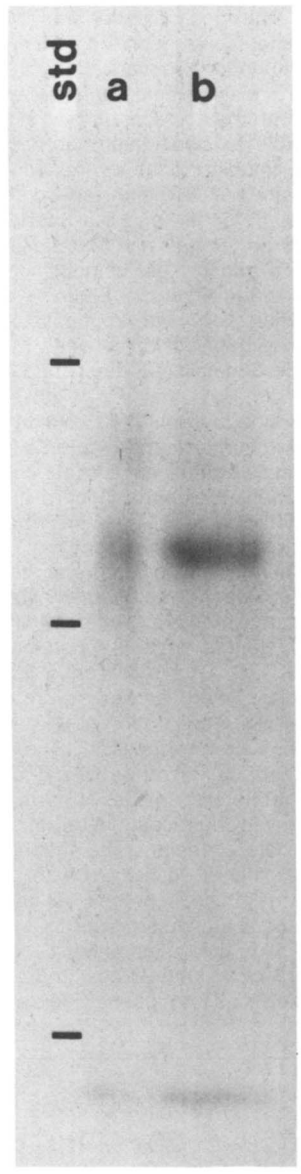

Figure 7. **SDS-PAGE analysis of purified K^k and D^d. Radioactive** material from elution A and C in Figure 6 was precipitated with cold acetone and subjected to SDS-PAGE. The gel was dried and autoradiographed. Standards are BSA, OVA, and Ribo A. *a*, H-2K^k from elution A; *b*, H-2D^d from elution C.

TABLE III

Binding of native or denatured H-2 molecules to MAb 1/42[a]

Sample	Cpm Bound (% of Applied)
β_2 Microglobulin	10
Heavy chain, H-2K^k	7
Heavy chain (K^k) + β_2 microglobulin	<1
H-2K^k	64

[a] ^{125}I-labeled H-2K^k was purified and treated with 6 M guanidine as described in the text. Heavy chain and β_2 microglobulin were separated by gel filtration, then dialyzed out of guanidine either separately or mixed together at a 2:1 (heavy chain:β_2-microglobulin) ratio. After dialysis, the samples were made 0.5% with Triton and directly applied to a 0.5 ml M1/42 column. The number of counts bound and eluted by DOC/0.65 M NaCl buffer are expressed as percent of applied counts.

2^d (both K- and D-ends) or purified H-2D^d (obtained from YAC cells; see Figs. 6 and 7) to M1/42 columns. At least 40 to 60% of these purified molecules re-bound to the MAb. Purified H-2^d proteins were also used to inhibit alloantisera-mediated complement cytotoxicity on appropriate cells (data not shown). By this method of assay, we found that at least 40% of the K^d and D^d serologic activity was recovered after purification.

The purified antigens also retained biologic activity. Purified H-2^d antigens, prepared by the protocol shown in Figure 3, were incorporated into lipid vesicles and used to stimulate a secondary allogeneic CTL reponse. Stimulation with liposomes containing 1 to 2 μg of H-2^d proteins resulted in a good response. We also tested the biologic activity of H-2^d antigens that eluted heterogeneously from M1/42 (see above, Fig. 4). A single preparation of P815 cells was loaded through M1/42 and sequentially eluted with 0.5% DOC (peak a), 0.25% DOC, 0.15 M NaCl (peak b), and DOC/0.65 M NaCl buffer (peak c). Material from each of the 3 peaks was incorporated into liposomes and used to stimulate an allogeneic CTL response. As shown in Figure 8, bottom panel, material from each of the peaks gave a distinct dose-response curve when assayed by CTL stimulation, with the 1st peak (eluted with DOC alone) yielding the least active material. CTL stimulation by the 2nd and 3rd peaks (b and c, Fig. 8 inset) compared favorably with stimulation by liposomes containing purified H-$2K^k$ (Fig. 8, top panel), where at least 75% of the original biologic activity is retained after purification (13, 21). The reason(s) for the different biologic activities of the 3 H-2^d fractions are not known; however, it does not appear to be due to separation of the K- and D-end molecules (see above).

DISCUSSION

M1/42, a rat anti-mouse MAb, binds to H-2 antigens of a variety of serologic specificities. We have used this antibody to quantitate and precipitate the H-2 antigens from the a, b, d, and k haplotypes. Most interestingly, M1/42, when coupled to Sepharose 4B beads, can be used as a highly effective affinity reagent. Affinity columns of M1/42 have been used to purify relatively large quantities of biologically active H-2 antigens from the b, d, and k haplotypes. Mild elution conditions (0.5% DOC and 0.65 M NaCl, pH 8) result in minimal damage to either antibody or antigen. Thus excellent yields of active antigen can be obtained and the MAb columns can be used repeatedly. M1/42 can also be used in series with a MAb 11-4.1 (αK^k) affinity column to isolate pure H-$2D^d$ from H-2^a cells.

M1/42 appears to recognize a species-specific, rather than allo-specific, antigenic determinant. Failure to bind β_2-microglobulin or isolated heavy chain provides preliminary evidence that M1/42 may recognize a determinant common to native, intact H-2 antigens. Our data indicate that M1/42 recognizes at least 1 and perhaps all 3 H-2 antigens from a number of haplotypes (Table I, Figs. 1 and 2). Specifically, binding to D^d and K^d has been shown by means of SDS-PAGE migration (Fig. 1) and, in the case of H-$2K^k$, by the successful binding of pure H-$2K^k$ to M1/42 affinity columns (Table III). We also have presumptive evidence that M1/42 recognizes H-$2D^k$, since the MAb can be used to purify a protein from RDM-4 cells that is not bound after several passages over an 11-4.1 column. It is possible, although unlikely, that M1/42 may recognize a small proportion of H-$2K^k$ that is partially denatured and therefore not retained by 11-4.1. However, our findings argue against this: separated H-$2K^k$ heavy chain or separated and reassociated H-2^k heavy chain and β_2-microglobulin do not bind to M1/42 (Table III), arguing that M1/42 is specific for determinants on native H-2 molecules. Furthermore, M1/42 precipitates a major band from AKR (H-2^k) spleen cell lysates that have been cleared of K^k (Fig. 1). It seems unlikely that such a large quantity of denatured H-$2K^k$ would be present in spleen cell lysates. It may be possible that M1/42 recognizes β_2-microglobulin, which would account for its wide specificity range. However, we have not found significant binding of separated β_2-microglobulin or β_2-microglobulin that has been denatured and partially reassociated with heavy chain (Table III).

Although not yet determined, it is possible that the product of the H-2L locus is also bound by M1/42. This is deemed likely because M1/42 has no apparent specificity for different H-2 antigens and because H-2L is structurally very similar to H-2K and -D (26). TI and Qa antigens also have some structural similarities to H-2, and it will be interesting to determine whether M1/42 binds these antigens. It should be noted that the tumor cell lines used as H-2 sources in the studies reported here do not bear TI or Qa.

M1/42 will be a useful reagent in a variety of immunologic studies. For instance, we have used M1/42 to quantitate H-2 on several cell types (Table II), and the results confirm the finding (13) that the tumor line RDM-4 expresses high levels of H-2^k. M1/42 can thus serve as a screening agent, both within and between haplotypes, for discerning fluctuations in H-2 expression. Such screening could be a useful adjunct to purification protocols, as evidenced by the abundant yield of H-$2K^k$ from RDM-4 cells (13). As shown in the gels in Figures 1 and 2, M1/42 allows easy comparison of H-2 antigens of different haplotypes, especially in cases where effective precipitating alloantisera are not available. An interesting example is that of the H-2^j haplotype, where an unusual ~38,000-dalton polypeptide was precipitated along with the expected 47,000 heavy chain (Fig. 2). M1/42 could be used to isolate larger quantities of the small polypeptide, allowing for further characterization of the molecule.

The wide specificity of M1/42 also makes it an excellent reagent for the purification of H-2 molecules, thus combining the ease, rapidity, and specificity of affinity chromatography with the wide applicability of conventional biochemical purifications. The conventional purifications of H-2 antigens that have been reported are lengthy, involving several column purifications (some requiring harsh elution conditions) and concentration steps, and result in rather poor yields of purified antigen (10–12). Using the procedure described in this report, several hundred micrograms of pure H-2 can be prepared in a 3- to 5-step procedure that involves very little loss of material and in which the rate-limiting operation is column-loading time. With respect to column loading time, we have found a good deal of variation between the viscosities of different whole cell lysates. Thus, H-2^b can be purified from whole cell lysates of EL-4 cells, whereas P815 cells, due to the viscous nature of the lysates, are usually fractionated before being solubilized and loaded onto the MAb column. Material bound to M1/42 can be eluted under very mild conditions, with 0.5% DOC and 0.65 M NaCl, pH 8.0, sufficient to remove the bound antigen (Fig. 4). Thus, the recovered antigens are exposed to a minimum of harsh treatment, can be readily dialyzed out of the elutant and into other buffers, and retain serologic and biologic activity. We estimate that D^d purified from M1/42 retains a minimum of 40% of its serologic activity, compared with the 1 to 10% recovery of H-2 typically found after laborious conventional purification methods (10–12).

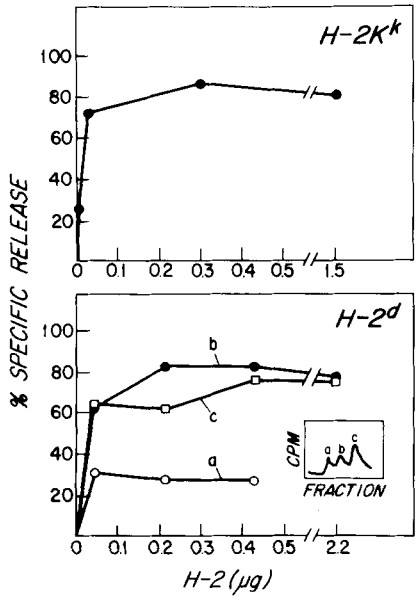

Figure 8. Stimulation of a secondary allogeneic CTL response by H-2-containing liposomes. H-$2K^k$ purified by affinity chromatography on MAb 11-4.1 was incorporated into liposomes as described (22) and various doses were used to stimulate primed $CD2F_1$ spleen cells as described in *Materials and Methods*. H-2^d from P815 whole cell lysates were purified on an MAb M1/42 column. Antigen was eluted in a stepwise fashion with 0.5% DOC (*peak a*), 0.25% DOC/0.15 M NaCl (*peak b*), and 0.5% DOC/0.65 M NaCl (*peak c*) in 20 mM Tris, pH 8. Material from each of the 3 peaks was incorporated into liposomes as described (22) and used to stimulate primed C57BL/6 spleen cells as described in *Materials and Methods*. CTL activity was assayed by using a standard ^{51}Cr release assay with RDM-4 (H-2^k) or P815 (H-2^d) cell targets.

Similarly mild elution of H-2 by DOC has been described for the 11-4.1 MAb affinity column (13). However, DOC did not elute HLA antigens from 6 different MAb affinity columns (Herrmann, Mescher, and Parham, unpublished), nor did it elute Ia molecules bound to a MAb (16; Turkewitz and Mescher, manuscript in preparation), indicating that elution by DOC is not a generalized property of MHC antigens.

H-2^d antigens obtained from P815 lysates have been found to elute in a heterogeneous manner (Fig. 5). This phenomenon is interesting but not easily explained. We have attempted to use DOC or salt gradient elutions to separate D^d and K^d molecules, and although there is some suggestion of selectivity in the elution, we were unable to obtain effective separation of the molecules in this way. The heterogeneous elution does appear to reflect differences in the molecules, as suggested by the biologic activities of the different peaks (Fig. 8), but it is not clear what this means, since the protein patterns from all peaks are similar.

One of the most interesting applications of this purification technology has been touched upon in this report: the biologic activity of the purified molecules. Using this pure material, it should be possible to investigate the functions of H-2 antigens in the many immunologic processes of which they are a part. This purification protocol makes available several H-2 antigens in quantities and conformations that are appropriate for functional, as well as biochemical, characterization.

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