Brief Definitive Report

THE INDUCTION OF SECONDARY CYTOLYTIC T LYMPHOCYTES BY SOLUBILIZED MEMBRANE PROTEINS*

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As a result of genetic and immunological evidence, it is now well established that the induction of murine cytolytic T cells (CTL) depends upon the recognition of antigens which are gene products of the major histocompatibility complex (MHC) of the stimulating cell. A more detailed understanding of the molecular events involved in this specific recognition would be greatly aided by the ability to obtain purified MHC antigens in a biologically active form. The serologically detectable MHC antigens are present on the surface of lymphocytes, and subcellular particulate preparations have been shown to retain activity for the induction of specific primary and secondary[1] (1–3) allogeneic CTL responses. We have demonstrated that this activity co-purifies with the plasma membrane fraction, and we have found no evidence for the involvement of soluble factors.[1] Because the antigens are membrane bound, the first step in their purification must be solubilization from the membrane by a method that does not result in loss of activity. Detergent solubilization of membranes has been used successfully for obtaining MHC antigens in serologically active form (4–6) and we demonstrate in this report that deoxycholate (DOC) can be used to obtain solubilized membrane proteins which retain activity for induction of a secondary allogeneic CTL response.

Materials and Methods

Mice. 6- to 12-wk-old mice of strains C57BL/6 (H-2^b) and DBA/2(H-2^d) were used in these studies.

Tumors. P815 (H-2^d) mastocytoma was maintained in DBA/2 female mice. EL4(H-2^b) leukemia was maintained in C57BL/6 mice.

Antiserum. Antiserum against H-2^d determinants was raised by hyperimmunization of C57BL/6 mice with P815 cells. The complement-lytic activity of this antiserum for P815 was removed by absorption with B10.D2(H-2^d), but not with B10(H-2^b) spleen cells (data not shown). This antiserum was the gift of Dr. R. Germain of the Department of Pathology, Harvard Medical School.

Generation of Effector Cells. C57BL/6 mice were immunized 4-6 wk before use by the

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intraperitoneal injection of 30×10^6 P815 cells suspended in Eagle's minimal essential medium or RPMI-1640. 7×10^6 spleen cells were cultured with varying amounts of membrane or solubilized membrane fractions in 2 ml of medium per 16-mm culture well (Linbro Chemical Co., New Haven, Conn.) in a humid atmosphere of 95% air and 5% CO_2. The medium consisted of RPMI-1640 (Grand Island Biological Co., Grand Island, N.Y.), 100 U of penicillin/ml, and 100 μg of streptomycin/ml, supplemented with 2 mM of glutamine, 5×10^{-5}M of 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum (Microbiological Associates, Walkersville, Md.). Membranes or solubilized fractions were added with rapid mixing after all other additions to the culture had been made.

^{51}Cr Release Assay. After 5 days of culture, cytotoxicity was assessed in a 4-h chromium release assay as previously described in detail (7). Percent of specific release is defined as E-C/FT-C (7).

Plasma Membrane Purification. Purification of the plasma membranes has been previously described in detail.[1] Briefly, tumor cells were lysed by nitrogen cavitation, and subcellular fractionation was carried out as described by Crumpton and Snary (8), with minor modifications. Purification, based on specific activity of 5'-nucleotidase, was 24- to 70-fold, and yields were 10–30%.

Detergent Solubilization of Membranes. Membrane solubilization was done using sodium deoxycholate (Sigma Chemical Co., St. Louis, Mo.) in 0.14 M of NaCl, 0.01 M of Na phosphate, pH 8 (phosphate-buffered saline; PBS). Membranes were incubated in DOC for 15 min at 0°C with occasional vortexing, and they were then centrifuged for 45 min at 100,000 *g*. The supernate containing solubilized proteins was removed and stored at 4°C until assayed. The DOC-insoluble pellet was resuspended in PBS, pH 8, and stored at 4°C. A detergent:membrane protein (wt/wt) ratio of at least 3–4 was required for maximum solubilization. Under these conditions, 75–80% of the membrane protein was present in the 100,000 *g* supernate. Detergent:protein ratios as high as 25 resulted in no additional solubilization. In the experiments described here, all solubilizations were done at detergent:protein ratios of 5 or greater, and a final DOC concentration of 0.5%. Protein was assayed by the method of Lowry et al. (9) using bovine serum albumin as the standard.

Sodium [^{3}H]deoxycholate (4 Ci/mM) was obtained from New England Nuclear, Boston, Mass. Protein A–Sepharose CL-4B and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Piscataway, N. J.

Results

Solubilization of CTL-Inducing Activity. We have previously shown that purified plasma membranes are able to induce a specific secondary allogeneic CTL response.[1] To determine if deoxycholate-solubilized membranes could be tested directly for stimulating activity, experiments were performed to determine what effect detergent would have on CTL induction by untreated membranes. DOC had little or no effect when added in amounts of up to 40 μg per culture, but at higher concentrations it became markedly inhibitory (data not shown).

DOC-solubilized membranes were able to stimulate a secondary CTL response (Fig. 1), although not as efficiently as whole membranes. Activity decreased above 5 μg of protein per culture due to the amount of detergent being added. The solubilized material retained activity after removal of the DOC by dialysis (>99.5% removal as determined in experiments using [^{3}H]DOC). Activity was approximately the same before or after detergent removal, but greater CTL responses could be generated with dialyzed material since it was possible to add more protein to the cultures without causing inhibition by detergent (Fig. 1). The decreased efficiency of solubilized material compared to whole membranes was not due to incomplete solubilization of the active molecules; a fact demonstrated by the inability of the detergent-insoluble pellet to induce a response (Fig. 1).

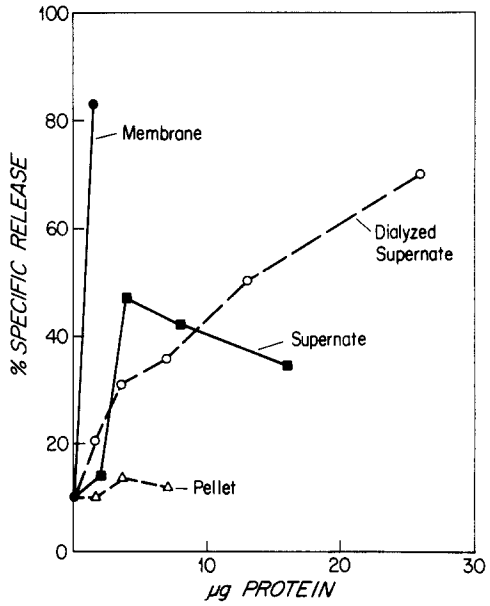

FIG. 1. Induction of cytolytic T lymphocytes by deoxycholate-solubilized P815 plasma membranes. Immune C57BL/6 splenocytes were cultured with membranes or solubilized fractions and cytotoxicity was assayed on P815 target cells as described in Materials and Methods. Specific release was determined at an effector:target ratio of 5. (●—●), whole plasma membrane; (■—■), DOC supernate; (○—○), DOC supernate after dialysis for 18 h against PBS, pH 8; (△—△), DOC-insoluble membrane fraction.

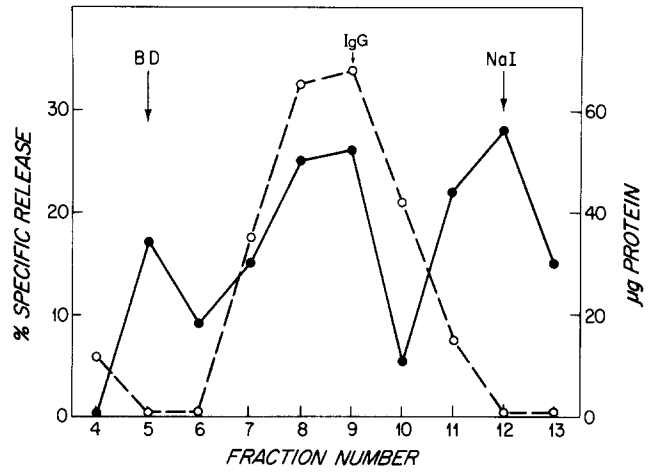

FIG. 2. Gel filtration of DOC-solubilized P815 membranes. Solubilized membrane protein (216 μg in 0.5% DOC) was chromatographed on a Sepharose 4B column (0.8 × 23 cm), and eluted with 0.1% DOC in PBS, pH 8. Fractions were assayed for CTL-inducing activity by incubating immune C57BL/6 splenocytes with aliquots of the fractions and assaying cytotoxicity on P815 target cells as described in Materials and Methods. Data shown were obtained using 50 μl of each fraction and an effector:target ratio of 25. Values for specific release have been corrected for background lysis occurring in cultures having no additions. (○- - -○), protein; (●——●), % specific release; BD, blue dextran.

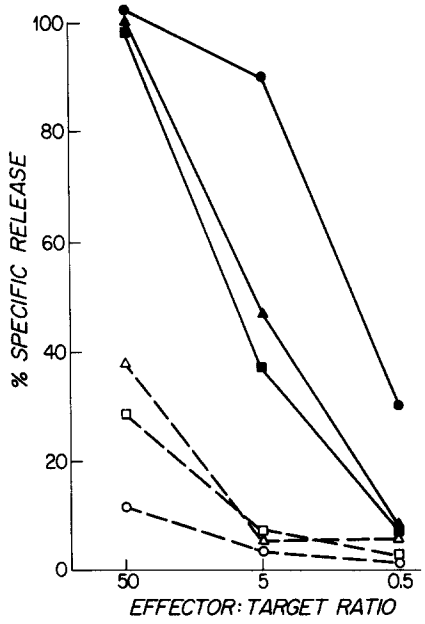

FIG. 3. Specificity of CTL induction by DOC-solubilized P815 plasma membranes. Immune C57BL/6 splenocytes were cultured with membranes or solubilized fractions and cytotoxicity assayed as described in Materials and Methods using P815 or EL4 as targets. (●), 1.5 μg whole membranes; (■), 4 μg DOC supernate; (▲), 6.6 μg DOC supernate after 18-h dialysis against PBS, pH 8; (——), P815; (- - -), EL4.

Failure of the CTL-inducing activity to sediment during centrifugation at 100,000 *g* suggests that it is in soluble form, but does not rule out the possibility of large protein or protein-lipid aggregates, or low density membrane fragments. Gel filtration of the solubilized material on Sepharose 4B in the presence of 0.1% DOC demonstrated that the majority of the activity was in a soluble form (Fig. 2). A small amount of high molecular weight activity was present in the excluded volume. The majority of the activity was present as two peaks, one eluting in the same region as human IgG (mol wt 156,000), and one of lower molecular weight. Detergent solubilization of H-2 (5, 10) and HLA (11) antigens under nonreducing conditions has been shown to result in the formation of some disulfide-linked dimers, and this may account for the occurrence of more than one peak of activity. This possibility is being further investigated.

Specificity of CTL Induction by Solubilized Membranes. Purified plasma membranes have been shown to retain specificity for induction of an allogeneic CTL response.[1] This was also the case for DOC-solubilized membranes, both before and after dialysis. Effector cells generated by stimulation with solubilized P815 membranes lysed P815(H-2^d), but not EL4(H-2^b) target cells (Fig. 3).

The induction of a specific CTL response by solubilized membranes would be expected to be the result of the soluble MHC antigens in the preparation. Evidence for this conclusion is provided by the demonstration that removal of the MHC antigens resulted in loss of stimulating activity (Table I). Addition of B6 anti-P815 (anti-H-2^d) antiserum to DOC-solubilized P815 membranes fol-

TABLE I

*Removal of CTL-Inducing Activity from DOC-Solubilized Membrane Proteins by B6 anti P815 (anti-H-2^d) Antiserum and Protein A-Sepharose**

Solubilized protein	Additions	Percent specific release	
		E/T‡ = 25	E/T = 12.5
μg			
0	–	38	18
5.4	Protein A-Sepharose	83	67
4.2	10 μl normal mouse serum + protein A-Sepharose	78	38
5.7	5 μl anti-H-2^d antiserum + protein A-Sepharose	29	15

* 50 μl (14 μg) aliquots of DOC supernate were incubated for 1 h at 4°C with or without serum. Protein A-Sepharose (10 mg solid in 100 ml PBS, pH 8) was then added and samples were again incubated for 1 h at 4°C with shaking. Samples were then centrifuged to remove protein A-Sepharose, and supernates were removed and assayed for CTL-inducing activity as described in Materials and Methods.

‡ E/T, effector:target ratio.

lowed by incubation with Protein A-Sepharose to remove antigen–antibody complexes resulted in complete removal of CTL-inducing activity.

Discussion

The results reported here demonstrate that MHC antigens retaining activity for induction of a CTL response can be obtained in a soluble form which should allow their further purification and study. There have been reports of subcellular fractions which retain CTL-inducing activity after treatments which might be expected to yield soluble products (3). Direct evidence has been lacking, however, to show that this activity is due to material that is in soluble form which will allow for further purification. It has also been reported that papain-solubilized, serologically active H-2 molecules are unable to induce a CTL response (1). We have similarly been unable to demonstrate CTL-inducing activity after solubilization with papain or Nonidet P-40 (unpublished results). Although all three methods yield soluble serologically active antigens, DOC solubilization might be effective, while papain and Nonidet P-40 are not, because of the antigens being presented in physically different forms (e.g. aggregates vs. single proteins) after addition to the culture. Alternatively, DOC solubilization might result in retention of molecular determinants which are necessary for CTL-inducing activity but not for serological activity, and which are lost upon Nonidet P-40 or papain solubilization. Further investigation will be necessary to determine which if either of these possibilities accounts for the apparent dissociation of serological and CTL-inducing activities. Similar explanations might account for the decreased efficiency of solubilized material in comparison to membranes. It should be noted that the question of whether or not DOC-solubilized material remains in soluble form after its addition to the culture remains to be answered.

The ability to obtain solubilized proteins active in induction of allogeneic CTL should make it possible to better understand the nature of recognition of foreign MHC antigens. In addition, if similar methods can be used to obtain soluble products active in CTL induction in systems involving recognition of chemically and virally modified syngeneic cells and syngeneic tumor cells, it will allow investigation of the molecular nature of these modifications in a manner not

otherwise possible. Attempts to obtain soluble materials active in these systems are in progress.

Summary

Membrane-bound antigens responsible for induction of a secondary allogeneic murine cytolytic T-cell (CTL) response have been obtained in a soluble, biologically active form by deoxycholate solubilization of tumor cell plasma membranes. The active proteins are soluble by the criteria of both ultracentrifugation and gel filtration. The immunological specificity of the induced CTL and removal of the activity from solution by treatment with B6 anti-P815 (anti-H-2^d) antiserum and Protein A-Sepharose demonstrate that the CTL-inducing activity is dependent upon solubilized major histocompatibility complex antigens.

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