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Immunogenicity of Subcellular Fractions and Molecular Species of MuLV-Induced Tumors

III. Stimulation of Syngeneic Antitumor Responses by Subcellular Fractions and Molecular Species of Moloney Virus-Induced Tumors in CBA and A Mice*

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Summary. *YBA, a Moloney virus-induced leukemia in CBA mice, and a relatively weak immunogenic tumor, was screened for the presence of immunogenic antigens. The tumor was subjected to homogenization and subcellular fractionation on sucrose gradients; the immunogenic subcellular fractions underwent further separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The immunogenicity of the subcellular fractions and the SDS-PAGE-isolated molecular species were tested by (their) subcutaneous injection into syngeneic mice and examination of their splenocytes examined against tumor cell and normal cell targets by the chromium release cell-mediated lympholysis assay. Tumor cell homogenates were also separated by SDS-PAGE and tested for immunogenicity without prior fractionation.*

Splenocytes from mice that had received injections of certain SDS-PAGE-isolated epitopes derived from YBA tumor homogenate or its light and heavy subcellular fractions generated effective cytotoxic responses against YBA target cells after 6 days in vitro cultivation. In contrast, intact YBA tumor cells or non-separated tumor homogenates failed to induce an efficient cytotoxic response. The effector cells induced with the immunogenic SDS-PAGE-isolated epitopes of YBA tumor were specific, since they cytolysed the homologous target cells more efficiently than unrelated target cells or syngeneic normal cells. The activity of these effector cells was affected by varying the effector : target ratio. Augmentation of the cytotoxic responses was obtained when the splenocytes of mice immunized with SDS-PAGE-isolated epitopes of YBA tumor were restimulated in vitro, with the homologous neoplastic cells.

Immunogenic SDS-PAGE epitopes were isolated from YAC tumor also (YAC is a Moloney-induced tumor of A mice). The effector cells induced with these separated epitopes were characterized as thymus-derived cells and not as natural killer cells.

The results suggest that (1) the molecular repertoire of YBA and YBA tumors contain immunogens that can induce a specific antitumor cell-mediated response; (2) the isolated molecular species injected are more efficient immunogens than the entire, unseparated homogenate sample or a dose of 10^8 intact inactivated tumor cells; and (3) the gel matrix may be responsible for the enhanced cell-mediated response induced against the weakly immunogenic tumor.

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Introduction

It might be legitimately assumed that tumor cells can be grown in a syngeneic host because these cells are non-immunogenic [9]. However, the fact that a tumor is non-immunogenic does not necessarily mean that such a tumor does not possess immunogenic potential. The immunogenic entities may be masked, or alternatively may be inactivated due to the co-existence of suppressogenic entities that induce dominant suppressor cells. The isolation of these hypothetical macromolecules carrying immunogenic determinants is the purpose of the present project.

YAC, a Moloney virus-induced tumor in A mice, is an example of a non-immunogenic tumor. It has been shown that a short in vitro cultivation of YAC cells converted these cells to be immunogenic [6]; this may be demonstrated by cell-mediated lympholysis induced against the non-immunogenic YAC target cells [6, 14]. Therefore displaying surface antigens recognizable by effector cells does not necessarily confer immunogenicity to these cells. The fact that the YAC tumor possesses immunogenic potential was further proved by our ability to isolate immunogenic entities from that tumor [14; present paper].

YBA, a Moloney virus-induced tumor of CBA mice, is also a weakly immunogenic tumor, although it is expected to express surface antigens such as the envelope antigen codes for the Moloney virus genome. The purpose of the experiments described here was to isolate immunogens from weakly immunogenic tumors and test their immunogenicity by monitoring their ability to induce cell-mediated responses in syngeneic hosts. This may be done by the separation of the plasma membrane, solubilization with a detergent, subjection of this solution to electrophoresis followed by a tedious extraction and dialysis procedure of many fractions, before they are injected into the mice. Since we needed a rapid screening method, an attempt was made to simplify this procedure. The tumor homogenate or its subcellular fractions were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the various gel slices were injected directly to different groups of mice to test their immunogenicity. Thus, protein purity and normalization of protein concentration among the various fractions was traded in favor of easily and rapidly obtaining information about the level and the specificity of the immune responses inducible by different tumor-cell entities. In addition, this method enabled

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us to examine our ability to immunize mice against a tumor and yet avoid the injection of intact tumor cells that, despite being inactivated, may constitute a potential of tumor development when injected to individuals.

This method was previously used to screen RBL5 cells for immunogens (RBL5 is a Rauscher virus-induced tumor of C57BL/6 mice [16]). The cellular material, except the nucleus, was subjected to SDS-PAGE so that the entire molecular repertoire was spread along the gel according to the apparent molecular weights, each 4 mm slice containing a variety of molecules with a similar apparent molecular size. It might be expected that intracellular proteins not expressed on the cell surface might break an existing immune tolerance towards their antigenic sites so that all the gel slices will become immunogenic; however, this has not been detected, probably because the cell surface antigens of the tumor target cells obviously interact selectively with effector cells that recognize only cell surface antigens, therefore acting as a probe. Thus only intracellular molecules that share common antigenic sites with surface molecules are expected to be inducers of target cell lysis. To ensure that intracellular public antigens common to many cells do not contribute to tumor cell lysis, syngeneic splenocytes were used as control target cells. Finally to detect more specific tumor antigens a histologically closely related tumor was used as for target cells also.

Materials and Methods

Mice. Male and female inbred CBA and A mice 8–12 weeks old were obtained from the animal colony of the Hebrew University – Hadassah Medical School.

Tumors. YBA, a Moloney virus-induced tumor of CBA mice (generously donated by Dr G. Klein of the Karolinska Institute, Stockholm), YAC, a Moloney virus-induced tumor of A mice [15] and RBL5, a Rauscher virus-induced tumor of C57Bl/6 mice [19] were maintained in ascitic form by weekly intraperitoneal passages of 10×10^6 cells in syngeneic hosts.

Culture Media. RPMI-1640 and fetal calf serum (FCS) were obtained from Bio Lab Ltd., Jerusalem. FCS was inactivated at 56° C for 40 min. HEPES buffer and 2-mercaptoethanol (2-ME) were obtained from Sigma Chemical Co., St Louis, MO, USA. Combined antibiotics were supplied by Bio Lab Ltd., Jerusalem, and contained per milliliter: penicillin 200,000 U, streptomycin 20,000 μg, neomycin 100,000 μg, and kanamycin 10,000 μg. The medium used for the in vitro experiments was RPMI-1640 supplemented with 15% FCS, 1×10^{-4} *M* 2-ME, 10–15 m*M* HEPES buffer, and 0.05%–0.1% combined antibiotics. This medium will be referred to as enriched RPMI medium.

One Way Mixed Leukocyte Tumor Cultures (MLTC). Cultures were set up in 50-ml tissue culture flasks (NUNC, Denmark) in a total volume of 15-ml. Each culture consisted of 30×10^6 responder spleen cells and 3×10^6 stimulator cells, or of 30×10^6 splenocytes of immunized mice. The stimulator tumor cells were first treated for 60 min at 37° C with mitomycin-C (mit-C) (Sigma, 7×10^7 cells in 4 ml of a mit-C solution at a concentration of 100 μg/ml) and then washed three times before being suspended in enriched RPMI medium. The cultures were prepared and incubated in an upright position in a humidified atmosphere of 5% CO_2/95% air. After 6 days, the cultures were terminated and the cells were pooled, centrifuged, washed and finally brought up to a concentration of 3×10^6 viable cells in RPMI medium supplemented with 10% FCS.

Mixed Lymphocyte Cultures (MLC). In the MLC, C57BL/6 responding allogeneic spleen cells were substituted for the CBA responding spleen cells. All the other technical procedures were the same as those for the MLTC.

Assay for Cell-Mediated Lympholysis (CML). The ^{51}Cr-release assay of Brunner et al. [3] was used with slight modifications. Twenty million target cells (1 ml) were radiolabeled by allowing them to react in test tubes with 200–300 μCi of ^{51}Cr (sodium chromate, The Radiochemical Centre, Amersham, England) for 40 min at 37° C. The test tubes were shaken occasionally during the incubation. The cells were then washed three times with 30 ml cold RPMI. Mixtures of 2×10^4 (0.2 ml) labeled target cells and 1×10^6 (0.3 ml) lymphocytes (giving an effector : target ratio of 50 : 1) were prepared in triplicates in 10×70 mm plastic serologic tubes. The tubes were centrifuged at 250 *g* for 2 min and then incubated for 4 h at 37° C in a humidified atmosphere of 5% CO_2/95% air. The reaction was terminated by addition of 1 ml cold medium. The cells were pelleted by centrifugation at 500 *g* for 10 min, and the supernatants were transferred to fresh tubes. Radioactivity in the supernatants was then determined in a gamma counter. The percentage of specific cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental } ^{51}\text{Cr release} - \text{Spontaneous release}}{\text{Total } ^{51}\text{Cr/tube} - \text{Spontaneous release}} \times 100 .$$

The experimental release was the amount of ^{51}Cr released from target cells incubated with sensitized cells. Spontaneous release was the amount of ^{51}Cr released from target cells. The total ^{51}Cr/tube, rather than values from freezing and thawing of labeled cells, was used in the formula, since some samples sensitized with allogeneic tumor cells gave almost 100% cytotoxicity. Spontaneous release ranged from 5%–15% of the total amount of the ^{51}Cr incorporated.

Cell Fractionation. Cellular fractionation of YBA cells was performed according to the method of Panet et al. (personal communication) as described below. YBA cells (200×10^6) were suspended in 1 ml homogenization buffer, pH 7.5, consisting of 10 m*M* Tris HCl, 10 m*M* NaCl, and 10 m*M* 2-ME, in an ice bath. The cells were then homogenized manually by 30 vertical strokes in a homogenizer sealed with a tight-fitting ground glass homogenizer. The unbroken cells (less than 1%) and the nuclei were removed by centrifugation for 10 min at 30,000 rpm in a PR-6000 IEC centrifuge. The supernatant was mixed with 60% sucrose in homogenization buffer, and the final sucrose concentration was brough up to 45% w/w.

Samples of the supernatant prepared in this way were incorporated into a stepwise sucrose gradient with concentrations of 20%, 25%, 30%, 45%, and 60% sucrose. Each gradient was spun for 16–18 h at 174,000 *g*, and the resulting sucrose gradient fractions were collected with the aid of a peristaltic pump (Gibson, France). The actual number of fractions collected depended on the aims of each set of experiments. Each fraction was suspended in 37 ml homogenization buffer and spun at 50,000 *g* for 90 min. The

supernatant was discarded and the pellet, i.e., the particulate cellular material, was suspended in 0.5 ml 0.01 *M* sodium phosphate buffer, pH 7.2. Samples of 0.2 ml of each suspension were injected SC into various groups of CBA mouse pairs. In some of the experiments the protein content of each fraction was measured by the method of Lowry et al. [18].

After 10–14 days the mice were killed, and the splenocytes of each mouse pair were pooled and cultured for 6 days in RPMI-FCS with or without mit-C-treated YBA stimulator cells. The incubated cells were then tested for CML by the ^{51}Cr-release assay against YBA, YAC, RBL5, and normal CBA ^{51}Cr blast splenocytes [the blast cells were induced with concanavalin A (con A); 30×10^6 cells in 15 ml enriched RPMI containing 2 μg/ml con A].

SDS-PAGE Analysis of the Tumor Homogenate and Its Sucrose Gradient Immunogenic Fractions. A homogenate of YBA or YAC or YBA sucrose gradient immunogenic fractions was treated with 2% SDS and 1% Triton X-100 for 30 min at room temperature. SDS-PAGE (7.5%) of the treated material was performed in cylindrical tubes under non-reducing conditions at 2 mA/gel for 16 h at room temperature according to the method of Weber and Osborn [21]. Each gel cylinder was loaded with 50–100 μg protein (derived from 5×10^6 cells). The following molecular weight markers were run simultaneously with the experimental gels: IgG 150 kilodalton (kd); human serum albumin (68 kd); ovalbumin (44 kd) and α-globin (15.5 kd). On termination of the electrophoresis the gels were divided into 4-mm slices that were injected into each of a different pair of syngeneic mice in a sequential order. The mice were killed 14 days later, and their splenocytes were cultured with or without a YBA sensitizer for 6 days before being tested for CML against the following ^{51}Cr-labeled target cells: YBA, YAC, RLB5, and normal CBA blast splenocytes. The percentage cytotoxicity was plotted against the number of the gel slice. This procedure is described in detail in one of our previous publications [13].

Cells Characterization. Thymus-derived enriched spleen cell populations were obtained by filtering 30×10^6 spleen cells through a nylon-wool column [10]. Spleen cells deprived of T cells were obtained by treatment with monoclonal anti-Thy-1 serum (diluted 1 : 500) and guinea pig complement, as previously indicated [17]. The anti-Thy-1 serum was generoulsy donated by Dr P. Lake, University College, London. The characterization of this antiserum has recently been published in detail elsewhere [17]. Cellular characterization was further performed by elimination of various subpopulations of T cells with the following different alloantisera and rabbit complement. The anti-Ly-2.2 and anti-Ly-3.2 monoclonal antibodies (diluted 1 : 20,000) and C3H/An anti-Ly-1.2 alloantiserum (diluted 1 : 20) were generously provided by Dr L. J. Old, Memorial Sloan Kettering Cancer Center, New York. Characterization of these alloantisera has been published by Yefenof et al. [22]. The anti-Ia^K alloantiserum (diluted 1 : 100 [1]) and the anti-I-J^K monoclonal antibody (diluted 1 : 100) obtained from newly developed hybridoma were provided by Dr M. Feldmann of University College, London. Natural killer (NK) cells were characterized by incubating the cells with anti-asialo GM1 antibody (diluted 1 : 50) and guinea pig complement [11, 12]. This antiserum was generously provided by Dr K. Okumura of the University of Tokyo, and ist anti-NK activity has been described in detail elsewhere [11, 12]. The guinea pig and rabbit complements were not cytotoxic to the cells when tested at the dilution (1/12) used for killing T cells in the presence of the various antisera.

Results

SDS-PAGE of YBA Tumor Homogenate

Spleen cells from CBA mice that had received mit-C-treated YBA cells did not generate cytotoxic responses when tested immediately after their removal from the mice. The non-immunogenicity of the YBA cells is in keeping with their high tumorigenicity: even doses as low as five viable YBA cells killed the host animals within 3 weeks (unpublished observations). Similarly, after 6 days of in vitro culture, spleen cells from mice that had received injections of mit-C-treated YBA cells mostly generated not more than 10% cytotoxicity (Fig. 1). The cytotoxic responses of in vitro-cultivated splenocytes from YBA-primed mice were determined in nine other experiments, and the cytotoxicity level rarely exceeded 12% (an exception is seen in the results described in Fig. 4). In contrast, Fig. 1 also shows that certain molecular species, i.e., gel slices 4, 5, 9, 15, 21, 25, and 28 (corresponding to molecular weights of 175, 100,

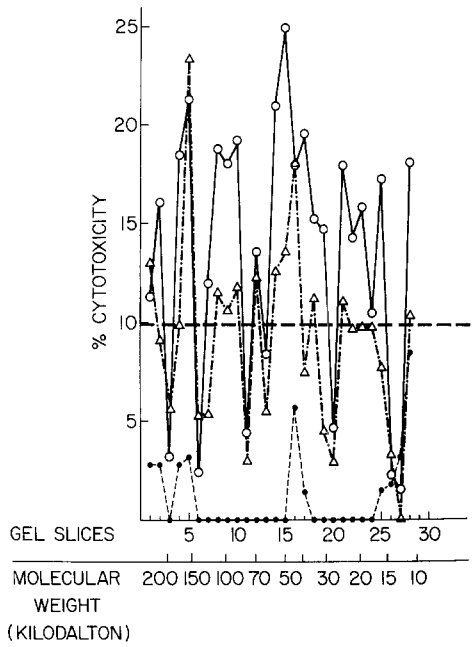

Fig. 1. Immunogenicity of SDS-PAGE slices of a YBA cell homogenate. YBA cells, 2×10^8, were homogenized and spun at 3,000 rpm for 10 min in a PR-6000 IEC centrifuge. The cellular material devoid of nuclei was treated with 1% Triton X-100 and 2% SDS and run on a 7.5% polyacrylamide gel. Portions (4 mm) of duplicate gels were injected into pairs of CBA mice in sequential order. Fourteen days later, the splenocytes of these mice were cultivated in vitro without stimulator cells. After 6 days, the cytotoxic responses were tested against different target cells: YBA, YAC and normal blast cells (derived from splenocytes stimulated with con A). The *horizontal line* indicates the cytotoxic response of splenocytes derived from mice injected with intact mit-C-treated YBA cells. An MLC of C57BL/6 responder spleen cells and YBA stimulator cells generated 54% cytotoxicity against YBA target cells. An MLC of C57BL/6 responder cells and normal CBA stimulator cells generated 57% cytotoxicity against normal CBA blast cells. Targets: (○———○) YBA; (△– · –△) YAC; (●– – –●) normal CBA blast cells

45–50, 27, 16, and 12 kd, respectively) that were isolated by SDS-PAGE of a YBA tumor homogenate induced immunoreactive cells in the spleens of CBA mice. These splenocytes generated a significantly higher level of cytotoxicity (18%–25%) than splenocytes derived from mice that had received intact YBA cells. Some of the molecular species induced effector cells that cytolysed both YBA and YAC target cells (gel slices nos. 5 and 16). These cytotoxic cells perhaps recognized antigens shared by both kinds of tumor cells. Other molecular species (i.e., gel slices 9, 21, 23, 25, and 28) induced effector cells that cytolysed the YBA tumor more efficiently than the YAC tumor, thus showing the specificity of these cytotoxic responses. Furthermore, effector cells induced with immunogenic molecular species of the YBA tumor homogenate failed to cytolyse normal CBA blast cells significantly (Fig. 1). These target blast cells were, however, cytolysed (57% cytotoxicity) by allogeneic effector cells generated by stimulation of C57BL/6 splenocytes with CBA mit-C-treated splenocyte stimulator cells, showing that the normal target cells were susceptible to lysis.

In the experiment documented in Fig. 1 we determined the cytotoxic responses generated by non-stimulated cultivated cells (the maximal cytotoxic response was 25% cytotoxicity: gel slice 15, Fig. 1). It was therefore of interest to determine whether addition of stimulator cells to the culture would improve the anti-YBA cytotoxic response.

Figure 2 demonstrates the cytotoxic responses of both non-stimulated (Fig. 2A) and YBA-stimulated (Fig. 2B) splenocytes from mice that had received SDS-PAGE isolated gel slices of a YBA tumor homogenate. Introduction of inactivated YBA stimulator cells into cultures of splenocytes derived from mice exposed to various molecular species augmented the cytotoxic responses of these splenocytes. For instance, gel slice 7 induced 20% cytotoxicity in non-stimulated splenocytes and 41% cytotoxicity in splenocytes exposed to stimulator cells. Similarly, gel slice 15 induced 17% cytotoxicity in splenocytes cultured alone and 52% cytotoxicity in splenocytes cultured with stimulator cells. In general, the immunogenic cytotoxic profile of the stimulated splenocytes was significantly stronger than that of the non-stimulated splenocytes. Once again, the experiment described in Fig. 2 indicates the specificity of the cytotoxic responses. Some of the immunogenic molecular species (e.g., gel slice 9, Fig. 2A; gel slice 7, Fig. 2B) generated significantly stronger cytotoxic responses against homologous target tumor cells (YBA) than against non-related tumor target cells (YAC or RBL5 tumor). Other molecular species generated cross-reactive cytotoxic responses, which were more pronounced in the stimulated than in the non-stimulated cellular cultures (e.g., gel slice 25, Fig. 2B). The strong cross-reactivity of the stimulated cultures was expressed largely against the YAC target cells and to a lesser extent against the RBL5 target cells. (It should be remembered that YBA and YAC tumors are both induced with the Moloney virus, whereas RBL5 is induced with Rauscher virus, and that the Moloney virus and the Rauscher virus are immunologically cross-reactive [7]). It should also be pointed out that gel slice 21 induced cytotoxic responses only against the non-related YAC tumor, in both the stimulated and the non-stimulated cultures (Fig. 2). Since the non-stimulated splenocytes from mice that had received immunogenic molecular species generated considerable cytotoxic responses, which were more specific than those of the stimulated splenocytes, we decided that we would limit out subsequent experiments to non-stimulated, rather than stimulated, splenocytes of the

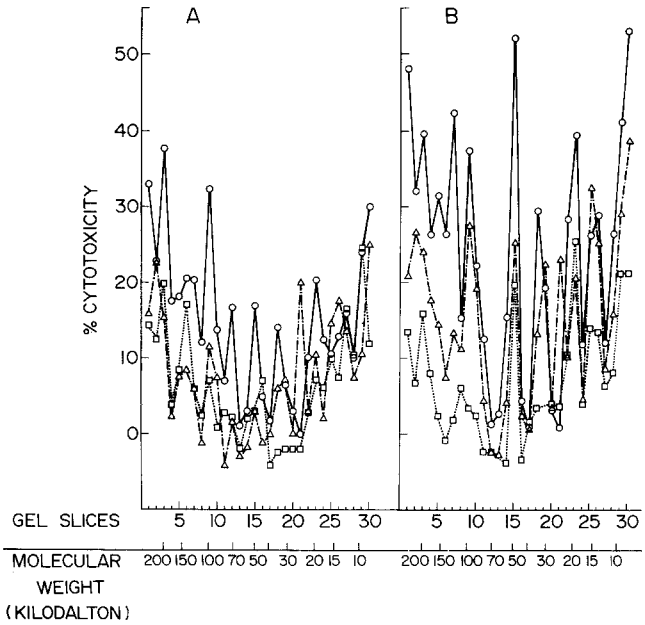

Fig. 2A and B. Cytotoxic responses of in vitro non-stimulated (**A**) and stimulated (**B**) splenocytes derived from mice injected with SDS-PAGE gel slices of a YBA tumor homogenate. The technical details are as described in the legend to Fig. 1, except that parallel splenocyte cultures (**B**) were stimulated with mit-C-treated YBA cells. Target cells were YBA cells. Targets: (○———○) YBA; (△– · – · –△) YAC; (□· · · · · □) RBL5

treated mice. In so doing, we also reduced the number of factors involved in our experimental system.

To evaluate further the significance of our results, we tested the effector cells induced with each of the different gel slices by varying the effector : target ratios (Fig. 3). Gel slices containing immunogenic molecular species (e.g., gel slice 13) induced cytotoxic responses in keeping with the various effector-target ratios, whereas non-immunogenic gel slices (e.g., gel slice 21) failed to induce cytotoxic responses at any effector : target ratio.

In order to address the question of whether the enhanced immunogenicity of the gels content is a purely quantitative phenomenon or whether other factors may be involved, some animals were immunized with YBA gel slices while others received aliquots of the low-speed-centrifugation supernatant of the YBA homogenate. These aliquots were equivalent to the samples that were run on SDS-PAGE; each one contained 64 μg protein. Some of the animals received 1 : 2 and 1 : 8 dilutions of these aliquots; the undiluted samples represent a yield of 5×10^6 YBA cell homogenate. In addition, 5×10^6 intact inactivated YBA cells were used as controls. Some mice also received blank gel slices, and others again were immunized with 100- and 200-fold larger amounts of cells. Figure 4 illustrates the cytotoxicity, 45%, 48%, 48%, induced by gel slices 14, 18, and 20, respectively, and the cytotoxicity induced by 10^8 intact YBA cells (28%) or 50 μl homogenate (23%; 64 μg protein). Since each of the gel slices contains only a fraction of the 64 μg protein originally applied to the gel it is unlikely that the immunogenicity of the gels is based exclusively on the antigenic quantity; it is probably amplified by the gel matrix that may serve as an adjuvant. The cytotoxic response induced with intact 10^8 YBA cells was relatively higher than that obtained in all our previous experiments (28%

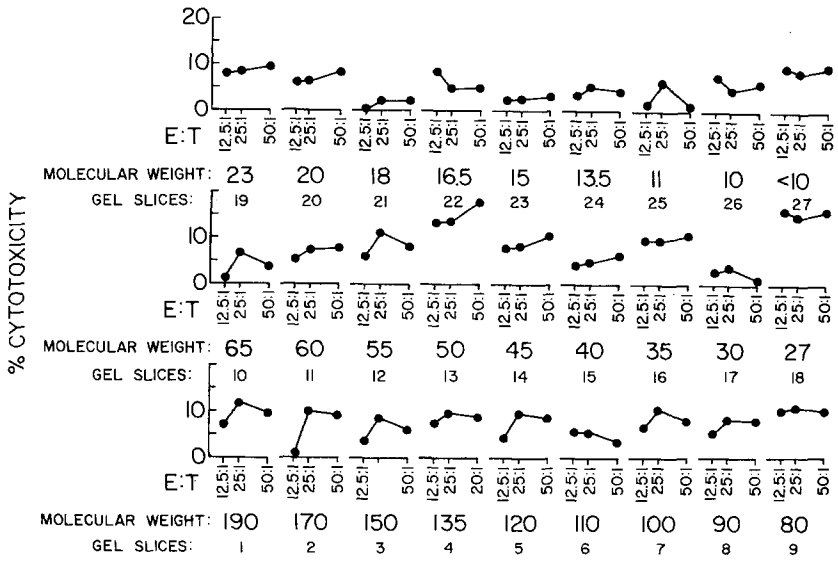

Fig. 3. Titration of the effector cells induced by immunogenic gel slices of SDS-PAGE of a YBA tumor homogenate. Cytotoxic responses were induced as described in the legend to Fig. 1. The cytotoxic responses were titrated by varying the effector : target (E : T) cell ratios. The target cells were YBA tumor cells

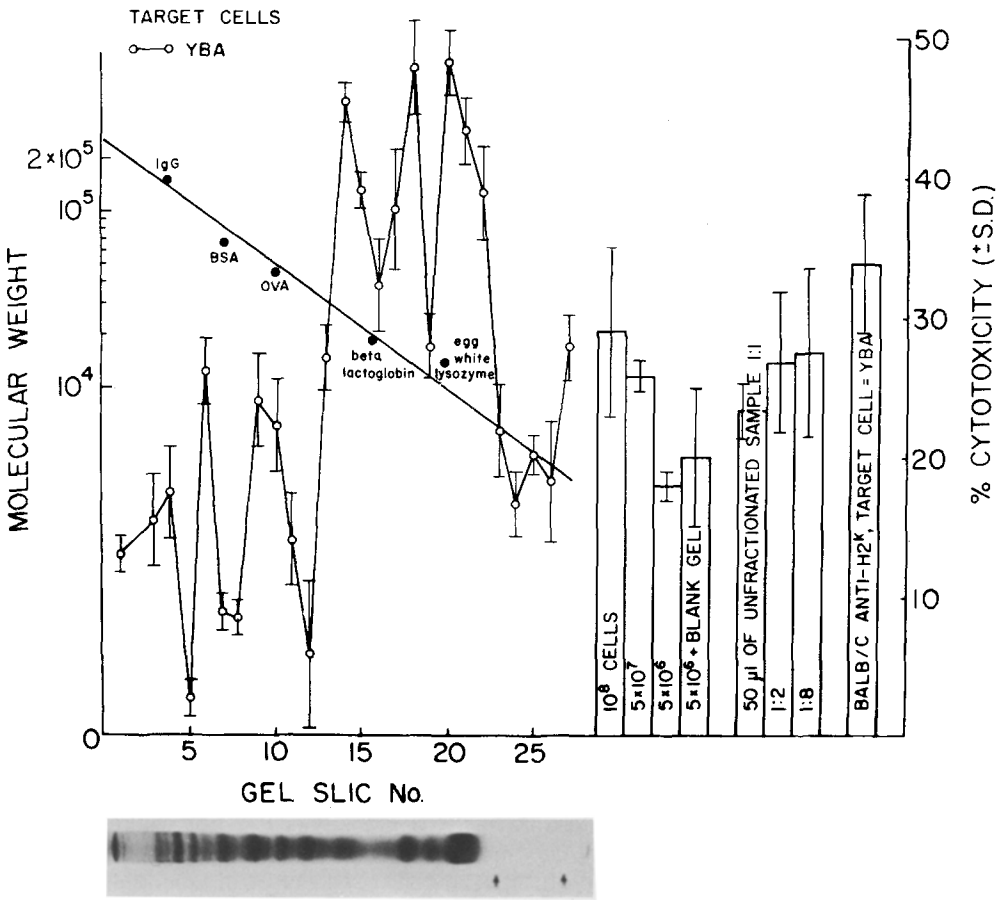

Fig. 4. Immunogenicity of SDS-PAGE slices of YBA cells versus immunogenicity of increasing intact YBA cell concentrations and unfractionated YBA cell homogenate. Cellular samples (50 µl) of YBA containing 64 µg protein each were treated as described in Fig. 1; 1 : 1, 1 : 2, and 1 : 8 dilutions of similar aliquots were injected into mouse pairs directly without electrophoresis. Intact inactivated YBA cells were also injected into other pairs of mice and the cytotoxicity induced was tested as in Fig. 1. The front dye was pyronin red, which migrated 16 mm ahead of the bromophenol blue and is used as mobility of 1.0. The stained gel is aligned with the corresponding injected gel slices at the horizontal axis; the *left arrow* indicates the front bromophenol blue dye and the *right arrow* designates the front pyronin red dye. An additional band was seen close to the front pyronin red dye when the gels were fixed with 10% trichloroacetic acid instead of the methanol-acetic acid fixer (data not shown)

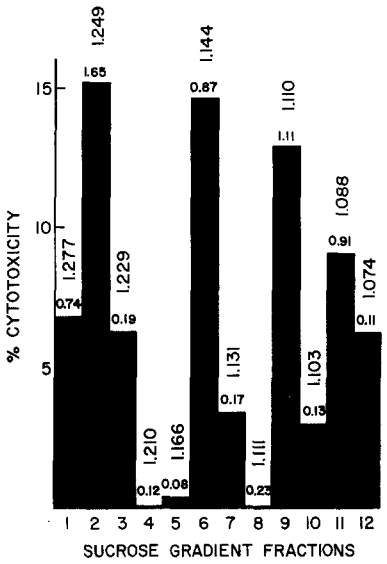

Fig. 5. Sucrose gradient fractionation of YBA cellular homogenate, the source for the light fraction, No. 9, described in legend to Fig. 6. YBA cells, 2×10^8, were homogenized and spun at 3,000 rpm for 10 min in a PR-6000 IEC centrifuge. The supernatant was run on a discontinuous 20%–60% sucrose gradient which became continuous (as determined by a Bausch & Lomb refractometer) during 16–18 h of centrifugation at 174,000 *g*. Twelve fractions were collected and the particulate material, which was obtained by suspending and then centrifuging each fraction for 90 min at 56,400 *g* was injected SC into CBA mice. After 10 days, splenocytes from each mouse were cultivated for a further 6 days without stimulator cells and were then tested for cytotoxicity against YBA target cells. Protein content (mg/ml) was measured in each fraction and is recorded horizontally on the top of each *bar*. [The density of each fraction (g sucrose/ml) is noted vertically on the top of each *bar*.] Splenocytes from normal CBA mice failed to generate syngeneic anti YBA cytotoxic responses 6 days after cocultivation with YBA stimulator cells. Target: YBA

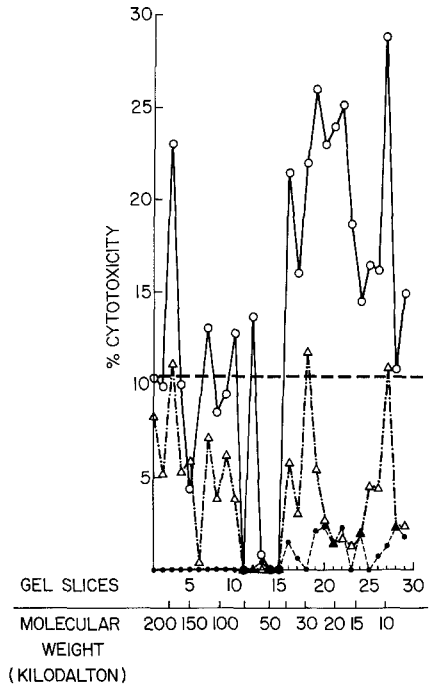

Fig. 6. Immunogenicity of SDS-PAGE gel slices of the light fraction of YBA cells homogenate. The immunogenic sucrose gradient light fraction (No. 9) of the experiment described in Fig. 5 was treated with 1% Triton X-100 and 2% SDS and then exposed to the technical procedure described in the legend to Fig. 1. The *horizontal line* indicates the cytotoxic response of splenocytes derived from mice injected with intact mit-C-treated YBA tumor cells. An MLC of C57BL/6 responder spleen cells and YBA stimulator cells generated 64% and 51% cytotoxicity against YBA and YAC target cells, respectively. An MLC of (57BL/6 responder spleen cells and normal CBA stimulator spleen cells generated 69% cytotoxicity against normal CBA blast cells. Targets: (O———O) YBA; (△– · –△) YAC; ●– – –●) normal CBA blast cells

cytotoxicity). However, it should be indicated that in this particular experiment all cytotoxic responses, including those induced with immunogenic molecular species (e.g., 45%–48% cytotoxicity), were stronger than usual. Figure 4 also presents the standard deviations of the cytotoxic responses. Similar deviations were obtained in all our other experiments.

SDS-PAGE of the Sucrose Gradient Light and Heavy Fractions of the YBA Tumor Homogenate

In order to improve the resolution of the immunogenic molecular species we decided to separate them from the tumor homogenate by a two-faceted procedure. In the first step, the YBA tumor homogenate was fractionated by sucrose gradient centrifugation, and the immunogenicity of each fraction obtained was measured with our standard test (determination of the cytotoxic responses developed by cultivated splenocytes from CBA mice that had received the different subcellular fractions). In the second step, light and heavy fractions were subjected to SDS-PAGE, as described above.

Fraction 9 (density of 1,110 g/ml; Fig. 5) was found to be the most potent fraction at the top of the gradient (13% cytotoxicity). This fraction, termed the light fraction, was further analysed by SDS-PAGE. The SDS-PAGE-isolated gel slices of fraction 9 (Fig. 5) were then injected into different groups of mice. The mice were killed 14 days later, and their splenocytes were cultivated for an additional 6 days before being tested for their ability to cytolyse various target cells. Figure 6 demonstrates that a high-molecular-weight species, gel slice 3 (200 kd), and various macromolecules with low molecular weights (gel slices 16 and 27) induced significant cytotoxicity responses (over 20% cytotoxicity), the highest response (30% cytotoxicity) being induced by gel slice 27 (10 kd). These cytotoxic responses were generated most effectively against the homologous YBA target cells, and less effectively against the non-related YAC target cells. Normal CBA blast cells almost completely failed to be cytolysed by the effector cells. However, both YAC cells and normal CBA blast cells were cytolysed efficiently by allogeneic anti-H-2^a and anti-H-2^k effector cells (51% and 69% cytotoxicity, respectively). The cytotoxic response induced with intact YBA cells was markedly less efficient (10% cytotoxicity) than the responses induced with the separated immunogenic molecular species.

In another experiment the sucrose gradient light fraction (no. 9, density of 1,110 g sucrose/ml) (Fig. 7) was electrophoresed simultaneously on two gel cylinders. One group of mice received gel slices of the first cylinder, and a second group of mice received gel slices of the second cylinder 1 day later (the second gel was preserved until injection at a temperature of −20° C). Figure 7 shows that the patterns of the immu-

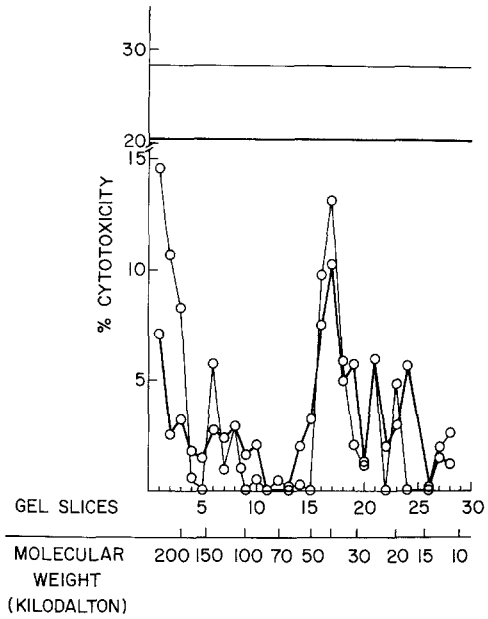

Fig. 7. Reproducibility of the immunogenic molecular species of YBA tumor light fraction. The immunogenic molecular species of a YBA light fraction were isolated by SDS-PAGE on two parallel gels. The SDS-PAGE-isolated gel slices of the two parallel runs were injected separately, with 1 day between, into two different groups of mice. The cytotoxic responses induced with the gel slices were recorded and are described on the figure by two separate *curves*. The *upper horizontal lines* describe the cytotoxicities developed against YBA target cells by MLC of C57BL/6 responder cells and YBA stimulator cells. Target: (O———O) YBA

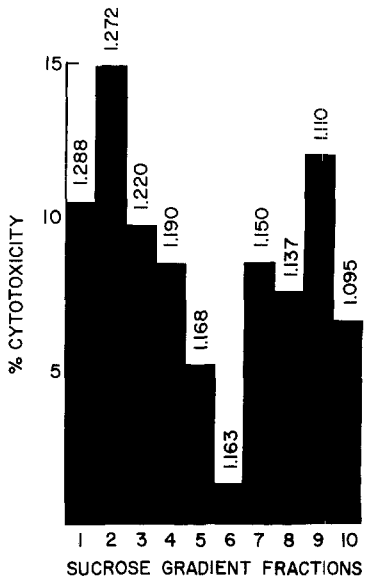

Fig. 8. Sucrose gradient fractionation of a YBA cellular homogenate – the source of the heavy fraction, No. 2, described in Fig. 9. Technical details as described in the legend to Fig. 5. The density of each fraction (g sucrose/ml) is recorded on the top of each *bar*. Target: YBA

nogenic molecular species of the two gels were similar, which strengthens the reliability of our methodology.

Sucrose gradient fraction no. 2 (density 1,272 g/ml) of the YBA homogenate that induced the highest cytotoxic response at the bottom of the gradient constituted the source of the heavy fraction (Fig. 8). This fraction was further analysed by SDS-PAGE (Fig. 9). Splenocytes from mice injected with the isolated 70-kd molecular species (gel slice 12) generated 19% cytotoxicity against the homologous YBA target cells, and 10% and 0% cytotoxicity against the YAC tumor and normal CBA blast cells, respectively. In contrast, allogeneic anti H-2^a and H-2^k effector cells significantly cytolysed these non-related target cells (81% and 78% cytotoxicity, respectively). Another molecular species (gel slice 19) also induced preferential cytotoxic responses against the homologous YBA tumor, while leaving the heterologous YAC tumor almost unaffected. Gel slice 14 induced similar cytotoxic responses against both YBA and YAC target cells. Effector cells induced with immunogenic molecular species of YBA cells almost completely failed to cytolyse normal CBA blast cells. While the 180 kd molecular species (gel slice 3) induced 26% cytotoxicity against YBA target cells, the intact YBA tumor induced only 12% cytotoxicity. In another experiment (complete data not shown) a different molecular species of the YBA heavy fraction (50 kd) induced 28% cytotoxicity against the YBA target cells, whereas under similar conditions the intact tumor cells induced only 2% cytotoxicity. In this experiment, the effector cells induced with the 50 kd molecular species completely failed to cytolyse normal CBA blast cells (1.5% cytotoxicity). These blast cells were, however, killed effectively by anti H-2^k allogenic effector cells (67.5% cytotoxicity).

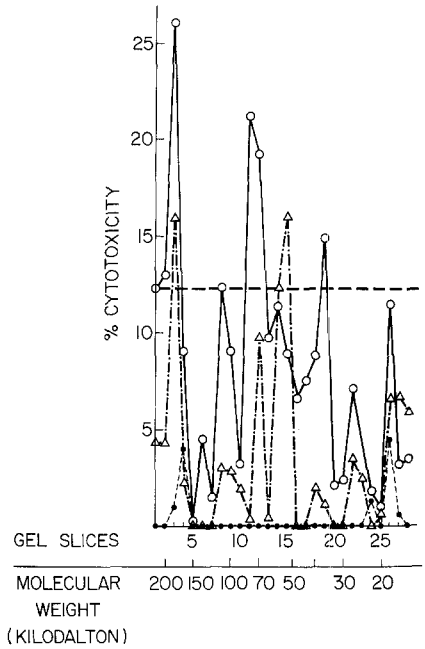

Fig. 9. Immunogenicity of SDS-PAGE gel slices of the heavy fraction of YBA cells. The immunogenic sucrose gradient heavy fraction, No. 2, of the experiment described in Fig. 8 was treated with 1% Triton X-100 and 2% SDS and then exposed to the technical procedure described in the legend to Fig. 1. The *horizontal line* indicates the cytotoxic response of splenocytes derived from mice that received intact mit-C-treated YBA tumor cells. Splenocytes from CBA mice that received mit-C-treated normal CBA splenocytes generated 9% cytotoxicity. An MLC of C57BL/6 responder spleen cells and normal CBA stimulator spleen cells generated 78%, 77%, and 61% cytotoxicity against normal CBA blast cells, YAC target cells, and YBA target cells, respectively. An MLC of C57BL/6 responder cells and YBA stimulator cells generated 78%, 81%, and 66% cytotoxicity against normal CBA blast cells, YAC target cells, and YBA target cells, respectively. Targets: (O———O) YBA; (△– · –△) YAC; (●– – –●) normal CBA blast cells

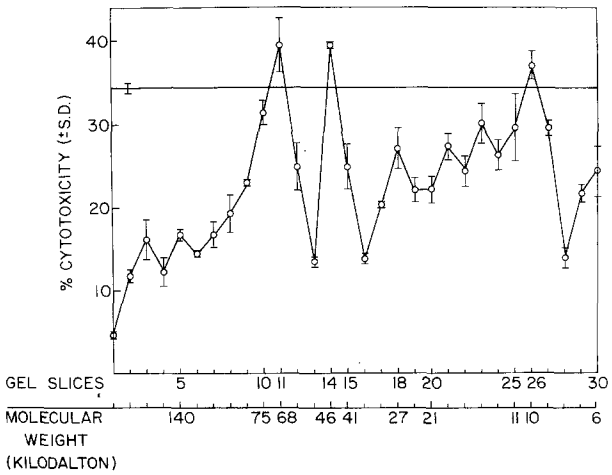

Fig. 10. Immunogenicity of SDS-PAGE gel slices of a YAC cells homogenate. A mice were injected with the SDS-PAGE gel slices. After 14 days the mice were killed and their splenocytes were cultivated for an additional 6 days. The cells were harvested at the end of the culture and their ability to cytolyse ^{51}Cr-YAC target cells was tested. The *horizontal line* indicates the anti-YAC cytotoxic response induced with the in vitro-cultivated YAC-1 cells. Target: YAC

SDS-PAGE of YAC Tumor Homogenate and Characterization of the Effector Cells Induced with the Immunogenic Entities

Our ability to isolate immunogenic entities from non-immunogenic tumor has been further demonstrated by experiments in which the YAC tumor was subjected to our experimental protocol. YAC, a Moloney virus-induced tumor of A mice, is an excellent subject for our experimental approach, since the intact inactivated tumor failed to stimulate the effector arm of the immune system [6], rather inducing very efficient function of suppressor cells [4]. In addition it should be indicated that YAC and YBA are closely related tumors, since both were induced by Moloney virus. Furthermore, the respective syngeneic hosts (A and CBA mice) share the left-hand side of the H-2 region.

Figure 10 confirms our previous observations [13, 14] by presenting evidence that certain SDS-PAGE-isolated molecular species (e.g., those with apparent molecular weights of 68 kd, 46 kd, and 10 kd) induced an efficient anti-YAC cytotoxic effector cells. In our previous studies we have shown that at least some of the isolated immunogenic entities induced cytotoxic effector cells that discriminated between the homologous tumor target cells and non-related target tumor cells,

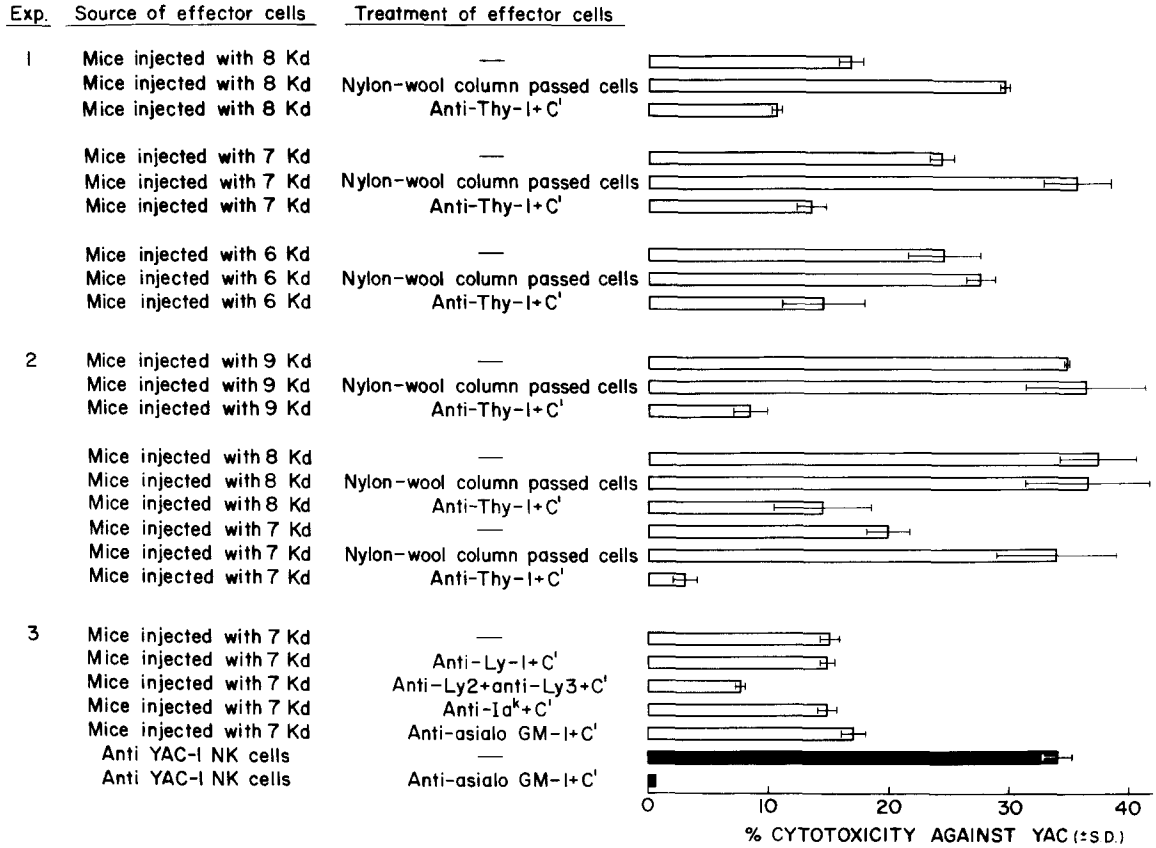

Fig. 11. Characterization of effector cells. Immunogenic molecular species of YAC tumor were isolated from the gels and injected into A mice. The mice were killed 14 days later and their splenocytes were removed, cultivated for an additional 6 days, and assayed for their ability to cytolyse radiolabeled target cells. The cellular sources of the effector cells were also treated as indicated in the figure. The various antisera were incubated with 2×10^6 for 30 min at room temperature, washed, and then reincubated for 45 min at 37° C with complement. The residual cells were counted and tested for their ability to cytolyse radiolabeled target cells. The anti-asialo GMI antibody and complement were also added to 3×10^7 C57BL/6 spleen cells and the activity of the NK cells was assayed against YAC-1 target cells *(black bars)*

whereas all the immunogenic epitopes induced effector cells that discriminated between the homologous target tumor cells and normal syngeneic target cells [13, 20]. We have further presented evidence that the in vitro-cultivated tumor, designated YAC-1, induced appreciable anti-YAC cytotoxic responses, whereas as mentioned before, the in vivo parent tumor failed to do so [6]. Indeed, Fig. 10 shows that the immunogenic SDS-PAGE-isolated molecular species of YAC tumor homogenate induced the same level of cytotoxicity as that induced with YAC-1 cells.

Our subsequent experiments were focused on the characterization of effector cells induced with SDS-PAGE isolated immunogenic entities of YAC tumor homogenate.

The effector cells induced with gel slices 28 (8 kd), 29 (7 kd), and 30 (6 kd) of the experiment described in Fig. 10 were passed through nylon-wool columns or exposed to anti-Thy-1 and complement. The treated cells were tested for their ability to cytolyse chromium-labeled YAC target cells. The nylon-wool-passed cells preserved their ability to cytolyse the target cells, whereas the anti-Thy-1-treated cells cytolysed the target cells less efficiently than the non-treated target cells (Fig. 11, expt 1). These results were confirmed by another independent experiment (Fig. 11, expt. 2). In another independent experiment (Fig. 11, expt. 3) it was found that effector cells induced with gel slice 29 (7 kd) were resistant to treatment with anti-Ly-1 serum and complement, anti-Ia^k serum and complement, or anti-asialo GM-1 serum and complement (anti-NK serum), but sensitive to treatment with anti-Ly-2 plus anti-Ly-3 sera and complement. The anti-asialo GM-1 serum and complement that failed to abrogate the activity of effector cells induced with isolated immunogenic entities completely blocked the activity of NK cells (Fig. 11, expt. 3). Thus the effector cells induced with isolated SDS-PAGE immunogenic entities present the expected markers of cytotoxic T cells ($Thy\text{-}1^+$, $Ly2^+3^+$, $Ly1^-$, Ia^-, asialo GM^-).

Discussion

YBA, a Moloney virus-induced tumor of CBA mice, is a relatively poor inducer of immune responses. In this respect, this tumor resembles a spontaneously induced neoplasm. We found that while YBA tumor rarely induced cytotoxic responses in excess of 10% cytotoxicity (an exception is seen in the results described in Fig. 4), various molecular species isolated either from the tumor homogenate or from its immunogenic subcellular fractions stimulated significant syngeneic cytotoxic responses that were two, three, or even ten times higher than those induced with intact tumor cells or their non-fractionated homogenates. Tumor homogenates at various doses also induced less efficient syngeneic cytotoxic responses than the isolated immunogenic molecular species derived from them (Fig. 4). Homogenate that was incorporated into the gel also failed to stimulate cytotoxic responses (data not shown). This finding emphasizes that the failure to detect immune responses in animals that had received injections of cells from a particular tumor does not necessarily mean that the tumor does not have an immunogenic potenital. Thus the implications of this observation are more than merely academic.

Our two-faceted methodology ensures that every population of immunogenic molecular species will be presented to the immune system. Furthermore, since the molecular species injected by us were incorporated into the gel slices, an adjuvant effect was probably also exerted on the immune system.

The sucrose gradient fractionation technique separates the different cellular components according to their relative densities. In the present study we did not attempt to characterize the sucrose gradient subcellular fractions, either quantitatively or qualitatively. Instead, we used the fractionation to achieve further resolution of the immunogenic molecular species and to ensure the presentation of intracellular immunogenic determinants to the immune system. We did not determine either the specific activities or the nature of each molecular species, since we focused our attention on the physical isolation of macromolecules carrying the immunogenic determinants and not on their quantitative or qualitative characteristics. It appears that the subcellular fractionation step before the SDS-PAGE may be omitted if the purpose of the SDS-PAGE is only to serve as a preparative screening step.

In our experiments, we selected representative sucrose gradient fractions which induced cytotoxic responses. We usually chose the most immunogenic fractions from the bottom (heavy fraction) and the top (light fraction) of the gradient. These sucrose fractions were further analysed by SDS-PAGE for the presence of immunogenic molecular species. The molecular species of an RBL5 tumor homogenate and its subcellular fractions were previously screened by the identical procedure [16]. In this earlier study, we partially characterized the sucrose gradient fractions of the RBL5 cellular homogenate by determining the protein content and the 5′ nucleotidase activity of each fraction. In our report, we presented preliminary evidence that the sucrose gradient light fraction of RBL5 was enriched with plasma membrane and we tentatively defined the heavy fraction as being enriched with insoluble intracellular particles [16].

Since precisely the same procedure was used in the present study, it is very likely that here also the light fraction presents immunogens of the membrane, whereas the heavy fraction presents immunogens of intracellular particles. However, since we isolated immunogens from normal CBA (unpublished results) and C57BL/6 [16] spleen cells also, and these immunogens induced cytotoxic responses that discriminated between syngeneic normal and tumor cells, it is possible that at least some of the immunogenic molecules are normal cellular material that is usually not exposed on the cell surface of normal cells.

Our methodology for isolating macromolecules carrying immunogenic determinants has some significant advantages. First, extraction procedures are completely avoided in both the sucrose gradient and the SDS-PAGE stages. Such extraction procedures may cause damage to the structure of the molecular species. Thus, we injected into the animals either non-extracted insoluble particles of the sucrose gradient fractions or molecular species incorporated into gel slices. Second, as previously mentioned, the fact that we did not extract the molecular species from the gel slices has another advantage, in that the matrix of the gel probably has an adjuvant effect. Blank gel slices that did not contain cellular material failed to induce non-specific immunological responses [13].

The cytotoxic immune responses of splenocytes derived from mice that had received immunogenic materials were tested after the cultivation of splenocytes and not after their immediate removal from the animals; evidence accumulated in our laboratory indicates that splenocytes from mice which were injected with immunogenic tumor cells [6] or their subcellular

fractions or molecular species [13, 14] failed to cytolyse the relevant target cells unless they were first cultured for several days in vitro. Cytotoxic effector cells appeared in such cultures even without the addition of stimulator cells. Similar observations have also been reported by other workers [2, 8]. The effector cells differentiated from the cultivated primed splenocytes were specific [5], and they were affected by varying the effector : target ratio [5]. Since in our hands this procedure provides the optimal, and sometimes the only, condition for generating cytotoxic cells while preserving the characteristics of immunological responses (i.e., specificity and sensitivity to secondary antigenic stimuli, see Fig. 2B), we decided to use it as a method for detecting cytotoxic reactions. The development of cytotoxic responses in the cultivated primed splenocytes were correlated in several cases, but not always]13], with the ability of the donor of these spleen cells to reject viable doses of malignant cells. The maximal cytotoxic responses generated by the non-stimulated primed splenocytes were in the range of 20%−30% cytotoxicity, whereas the maximal cytotoxic response of stimulated primed splenocytes was 50% cytotoxicity. Thus, the cytotoxic response of the non-stimulated splenocytes is moderate, although undoubtedly significant. However, it should once again be pointed out that these syngeneic cytotoxic responses were induced by molecules of tumors that are themselves very poor inducers of immune reactions.

Any attempt to compare comprehensively the immunogenic molecular species of the cellular homogenate with those of its subcellular fractions, or to compare the molecular species of the heavy fraction with that of the light fraction, is premature. We should remember that some molecular species may contain several populations of macromolecules with identical molecular weights. Furthermore, some of the molecular species are perhaps splitting products of larger macromolecules. Thus, to refine the methodology, we must characterize each immunogenic molecular species by means of biochemical and immunological techniques. In so doing, we will be able to characterize the macromolecules carrying immunogenic determinants and to ascertain whether they are of viral or cellular origin.

Another technical problem that still requires experimental elucidation is the improvement of our ability to isolate the same immunogenic components at different experiments. Although Fig. 7 shows that immunogens obtained from the same electrophoresis runs, but injected into two separate groups of animals at different times, induced the same immunological profiles, it was more difficult (but not impossible, [16]) to isolate the same immunogens from different cellular fractionations (compare Fig. 5 with Fig. 8) or electrophoresis runs (compare Fig. 1 with Fig. 2). We assume that this difficulty is caused by the fact that the cell cycle of the tumor cells is different from one experiment to another, thus expressing different antigenic presentation. Synchronizing the cell cycles of both the immunizing cellular material and the target cells may provide the only approach to overcome this problem.

The cytotoxic responses induced with some of the immunogenic molecular species derived from the YBA tumor were expressed maximally against YBA target cells, and to a lesser degree against YAC and RBL5 target cells. Other immunogenic molecular species induced cytotoxic responses that were expressed equally against the YBA cells and the non-related target neoplastic cells. YBA and YAC are closely related tumors, since both were induced with the Moloney virus. In addition, these tumors shared the left-side product of the H-2^k genetic complex. The RBL5 tumor also shares antigens with YAC and YBA cells, since the Moloney virus and the Rauscher virus cross-creact immunologically [7]. Despite these close antigenic relationships, some molecular species of the YBA tumor induced a significantly preferential cytotoxic response against the YBA target cells. This fact indicates that at least the portion of the molecular species which contains the specific antigenic structure was not destroyed during the SDS-PAGE separation procedure. Our previously published data are in agreement with this argument. For instance, in one of our previous studies [16] we demonstrated that in syngeneic C57BL/6 mice certain SDS-PAGE molecular species of RBL5 tumor induced preferential cytotoxic responses against RBL5 target cells but not against YAC target cells or 136.4B target cells (136.4B is a radiation-induced tumor of C57BL/6 mice).

The fact that we were able to isolate potent immunogenic entities from another non-immunogenic tumor, YAC (Fig. 10), strengthens our argument that even non-immunogenic or poorly immunogenic tumors may contain an efficient immunogenic potential that can be revealed after experimental manipulations. Using a similar technical approach to detect immunogenic potential in spontaneous tumors is obviously an immediate goal for our future studies. The YAC experimental model also presents clear evidence that the isolated immunogenic molecular species induce the appearance of cytotoxic T cells and *not* NK cells. Using various alloantisera and complement we have shown that the effector cells present markers of cytotoxic T cells but not those of NK cells.

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References

1. Beverley PCL, Woody J, Dunkley M, Feldmann M (1976) Separation of suppressor and killer T cells by surface phenotype. Nature 262: 495
2. Boon T, Van Snick J, Van Pel A, Uyttenhove C, Merchand M (1980) Immunogenic variants obtained by mutagenesis of mouse mastocytoma P815. II. Lymphocyte-mediated cytolysis. J Exp Med 152: 1184
3. Brunner KT, Mauel J, Cerottini J-C, Chapuis B (1968) Quantitative assay of the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. Immunology 14: 181
4. Devens B, Galili N, Deutsch O, Naor D, Klein E (1978) Immune responses to weakly immunogenic virally induced tumors. II. Suppressive effects of the in vivo carried tumor YAC. Eur J Immunol 8: 578
5. Devens B, Schochot L, Naor D (1979) Immune responses to weakly immunogenic virally induced tumors. V. Short in vitro cultivation of YAC changes its antigenic properties. Cell Immunol 44: 442
6. Galili N, Devens B, Naor D, Becker S, Klein E (1978) Immune responses to weakly immunogenic virally induced tumors. I. Overcoming low responsiveness by priming mice with a syngeneic in vitro tumor line or allogeneic crossreactive tumor. Eur J Immunol 8: 17
7. Glynn JP, McCoy JL, Fefer A (1968) Cross-resistance to the transplantation of syngeneic Friend, Moloney and Rauscher virus-induced tumors. Cancer Res 28: 434

8. Goldstein P, Luciani M-F, Wagner H, Rollinghoff M (1978) Mouse T cell-mediated cytolysis specifically triggered by cytophilic xenogeneic serum determinants: a caveat for the interpretation of experiments done under 'syngeneic' conditions. J Immunol 121: 2533
9. Hewitt HB, Blake ER, Walder AS (1976) A critique of the evidence for active host defense against cancer, based on personal studies of 27 murine tumors of spontaneous origin. Br J Cancer 33: 241
10. Julius MH, Simpson E, Herzenberg LA (1973) A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur J Immunol 3: 645
11. Kasai M, Iwamori M, Nagai Y, Okumura K, Tada T (1980) A glycolipid on the surface of mouse natural killer cells. Eur J Immunol 10: 175
12. Kasai M, Yoneda T, Habu S, Maruyama Y, Okumura K, Tokunaga T (1981) In vivo effect of anti-asialo GM1 antibody on natural killer activity. Nature 291: 334
13. Klein BY, Frenkel S, Ahituv A, Naor D (1980) Immunogenicity of subcellular fractions and molecular species of MuLV-induced tumors. I. Screening of immunogenic components by isopycnic ultracentrifugation and polyacrylamide electrophoresis of a tumor homogenate. J Immunol Methods 38: 325
14. Klein BY, Devens B, Deutsch O, Ahituv A, Frenkel S, Kobrin BJ, Naor D (1981) Isolation of immunogenic and suppressogenic determinants of the nonimmunogenic YAC tumor and the change in its immunogenic repertoire after in vitro cultivation. Transplant Proc 13: 790
15. Klein G, Klein E, Haughton G (1966) Variation of antigenic characteristics between different mouse lymphomas induced by the Moloney virus. J Natl Cancer Inst 36: 607
16. Kobrin BJ, Naor D, Klein BY (1981) Immunogenicity of subcellular fractions and molecular species of MuLV-induced tumors. II. Stimulation of syngeneic anti-tumor cell mediated immune responses by subcellular fractions and molecular species of Rauscher-virus-induced RBL5 tumor. J Immunol 126: 1874
17. Lake P, Clark EA, Khorshidi M, Sunshine GH (1979) Production and characterization of cytotoxic Thy-1 antibody-secreting hybrid cell lines. Detection of T cell subsets. Eur J Immunol 9: 875
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265
19. McCoy JL, Fefer A, Glynn JP (1976) Comparative studies on the induction of transplantation resistance in BALB/C and C57BL/6 mice in three murine leukemia systems. Cancer Res 27: 1743
20. Sharon R, Klein BY, Naor D (1982) T cell and cancer. Proceedings of the XIth Triennial World Congress of Pathology (in press)
21. Weber K, Osborn M (1975) Proteins and sodium dodecyl sulfate: molecular weight determination on polyacrylamide gel and related procedures. In: Neurath H (ed) The proteins. Academic Press, New York, p 179
22. Yefenof E, Azar Y, Eidelsztein P, Ben-Sasson SZ (1982) Immortalization of antigen-specific helper T cell lines by transformation with the Radiation Leukemia virus (RadLV). J Immunol 128: 625

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