Cancer Immunol Immunother (1988) 27: 142–146

Cancer Immunology Immunotherapy

Recovery of a cell surface fetal antigen from circulating immune complexes of melanoma patients*

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Summary. A well-characterized 69.5×10^3 dalton glycoprotein fetal antigen (FA), isolated from the spent culture medium of a melanoma cell line, UCLA-SO-14 (M14), was utilized to characterize the antigen component of circulating immune complexes (CIC) from melanoma patients. Ten serum samples from five patients with stage II melanoma at 1 and 4 months prior to the clinical detection of recurrent disease were selected for study. The CIC were dissociated with low pH and ultrafiltered through a 100×10^3 dalton exclusion limit membrane. The low pH treatment resulted in an increase in antibody titer in eight of ten serum samples. The antibody activity in membrane immunofluorescence was quantitatively inhibited by the filtered antigen fraction and purified FA, suggesting the presence of anti-FA antibodies in the treated serum, which possibly were complexed with FA in the untreated sample. As determined by competitive inhibition in an enzyme-linked immunosorbent assay, the filtrate (antigen fraction) contained an antigen that was immunologically similar to FA. These results clearly demonstrate that FA, expressed on the cell surface of melanoma cells, is present in CIC of selected melanoma patients.

Introduction

Since the initial description of circulating antigen-antibody (immune) complexes (CIC) in cancer by Sjogren et al. [30] and subsequently by Klein et al. [22], it has become apparent that CIC are a consistent feature of neoplasia. It is not unreasonable to expect that some of the antigens present in CIC are tumor-associated. For this reason, determination of CIC levels has been proposed both to monitor the effects of therapy [25, 27] and for immunoprognosis [11, 16]. However, CIC in an individual may arise from sources other than tumor activity. Antibody [19], antiidiotype [5, 15, 23] and other altered self components [2, 6, 18] have been detected in CIC of cancer patients. Since most CIC detection assays are antigen nonspecific, it is not surprising that the clinical utility of CIC analysis in human cancer has been controversial and limited [6, 28]. The ability to determine the presence of tumor-associated antigen(s) (TAA) in CIC should improve their clinical utility. However, progress in defining the nature of the antigen(s) present in CIC has been slow. This is mainly due to the unavailability of well-defined antigens that are recognized by antibodies in the sera of melanoma patients.

We have demonstrated the presence on melanoma cells and subsequently isolated two distinct antigens from the spent culture medium of a melanoma cell line, UCLA-SO-14 (M14) [12]. Both antigens were defined using antibody from sera of melanoma patients. One antigen is associated with melanoma and has been designated as melanoma TAA [9] while the second antigen appears to be of fetal origin. This fetal antigen (FA) [8] is a glycoprotein with a molecular weight of 69.5×10^3 daltons. Our initial studies have shown FA to be both heat stable and resistant to changes in pH. The antigenic activity of this 69.5×10^3 dalton glycoprotein FA resides in the carbohydrate portion of the molecule. Further studies on the treatment of FA by various immobilized lectins and enzymes demonstrated that B-D-galactoside or its derivative is one of the key constituents of the determinant recognized by antibodies present in sera of melanoma patients.

The FA is widely distributed in tumor tissues of various histologic types including melanoma, sarcoma, and carcinoma but in only 5% of tissue from normal individuals [8]. For this reason, monitoring FA may be useful in following responses to therapy or for immunoprognosis. Because 63% of melanoma patients have antibodies of the IgG class and 81% of the IgM class to FA [8], we hypothesized that FA, if present in serum, would be in the form of CIC. Our initial studies of eluates of protein A-positive *Staphylococcus aureus* cells used for extracorporeal immunoadsorption treatment of melanoma patients suggested that an antigen, immunologically similar to FA was present in the isolated IC [13]. The present investigation was undertaken to determine if FA or immunologically similar antigen could be detected in IC or similar material in sera from melanoma patients. For this purpose serum samples from melanoma patients were acid treated and ultrafiltered. The retained material was tested for antibody activity to melanoma cells by membrane immunofluorescence and filtered material for FA activity by a competitive enzyme-linked immunosorbent assay (ELISA). Results described here clearly establish that some of the CIC present in serum from melanoma patients contained FA and the corresponding antibody.

* Supported in part by NIH grants CA 30019, CA 12582, CA 09010, CA 29605 awarded by DHSS

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Materials and methods

Serum. We have previously demonstrated that patients with an intermediate tumor burden have the highest CIC levels [25]. For this reason, 10 serum samples from 5 patients with stage II melanoma at 1 and 4 months prior to the development of recurrent disease were selected. Sera were stored at −37° C until tested.

Cell line. A melanoma cell line, UCLA-SO-M14 (M14) was established from a biopsy specimen of a male patient with blood type O. The cells were maintained in RPMI 1640 medium (Flow Laboratories, McLean, Va.) supplemented with 10% fetal calf serum and antibiotics (100 IU penicillin, 100 μg streptomycin, and 5 μg Fungizone/ml). M14 from passages 18–20 were utilized as targets in indirect membrane immunofluorescent (IMIF) studies.

Preparation of FA. The FA was isolated and purified from the spent culture medium of the M14 cell line which had been adapted to grow in a chemically defined medium [3]. Details of the collection and preparation of the spent culture medium have been previously reported [8]. Briefly, the spent chemically defined medium was concentrated and ultrafiltered through a membrane with an exclusion limit of 100×10^3 daltons. The material retained on the membrane was chromatographed on a Sepharose 6B column and the antigen fraction was extracted with chloroform:methanol (2:1). The aqueous phase contained FA.

Dissociation of IC. A modification of the method originally described by Sjogren et al. [30] and more recently applied by Kirkwood and Vlock [14], was utilized to dissociate CIC. Serum (3 ml) was added to 50 ml of 0.1 *M* glycine:HCl buffer (pH 3.1) in an ultrafiltration chamber fitted with a YM 100 membrane (Amicon Corp., Danvers, Mass.). Ultrafiltration was performed at 4° C under 60 psi of nitrogen. The retained material was washed three times with 0.25 *M* phosphated-buffered saline (pH 7.2) (PBS) and reconstituted to its original volume for use in IMIF. The material filtered through the YM 100 membrane (100×10^3 dalton exclusion limit) was ultrafiltered on a membrane with a 10×10^3 dalton exclusion limit, washed with PBS (pH 7.2), and concentrated 6-fold to a volume of 0.5 ml. This fraction, designated the antigen fraction, was used as an inhibitor in IMIF and ELISA.

Indirect membrane immunofluorescence. The details of IMIF have previously been published [21]. Cultured cells were harvested by treatment with versene ($\times 10^{-4}$ *M* sodium EDTA, 0.14 *M* NaCl, 5×10^{-5} *M* KCl, and 5×10^{-3} *M* dextrose) and trypsin. The harvested cells were washed three times with Hanks balanced salt solution (HBSS) containing 0.01% human serum albumin and resuspended in albumin-supplemented HBSS at a concentration of 1×10^6 cells/ml. The IMIF was performed as follows: 50 μl of cell suspension was mixed with 50 μl of melanoma serum (both before and after acid dissociation and ultrafiltration) and incubated at 37° C for 1 h and at 4° C for 1 h. The cells were washed three times with albumin-supplemented HBSS and reacted with 50 μl of fluorescein-conjugated goat anti-human IgG and IgM at 23° C for 20 min. After washing, the cells were resuspended in 25 μl of 50% glycerol in 0.025 *M* PBS (pH 7.2), placed on glass slides, and examined for membrane immunofluorescence under a fluorescent microscope. Three or more fluorescent dots on the cell membrane were considered positive for the presence of antibody. Diffuse or cytoplasmic fluorescence was not regarded as positive. The percentage of positive cells was calculated following a 50 cell count. Greater than 10% positivity above controls was considered positive for the presence of antibody. Diffuse or cytoplasmic fluorescence was not regarded as positive.

To determine antibody specificity, 50 μl (87.5 mg protein) of purified FA was mixed with 50 μl of serum (before and after acid dissociation and ultrafiltration) and incubated at 37° C for 1 h and then 4° C for 1 h. The inhibited serum was then utilized in IMIF as previously described.

Competitive ELISA. A competitive ELISA was utilized to determine the presence of FA in the filtered material. Immulon microtitration plates (Dynatech Laboratories, Alexandria, Va.) were sensitized with 100 μl of purified FA (175 ng protein) diluted 1:50 with 0.06 *M* carbonate buffer (pH 9.6), incubated at 37° C for 3 h, and stored at 4° C until used. Prior to use, the wells were emptied and washed three times with 0.25 *M* sodium phosphate buffer supplemented with 0.15 *M* NaCl and 0.05% Tween 20. The test samples were placed in a boiling water bath for 3 min and the antigen(s) extracted with PBS followed by centrifugation at 7000 *g* for 10 min. Serial doubling dilutions of the test sample were mixed with equal volumes of an allogeneic control serum, known to have a high anti-FA antibody titer, and incubated at 37° C for 1 h, then 100 μl of each mixture was dispensed into wells and incubated at 37° C for 1 h. The binding of antibodies to FA absorbed onto the plates was determined by goat anti-human IgM conjugated to alkaline phosphatase (Sigma Corp), with *p*-nitrophenyl phosphate (1 mg/ml) in 10% diethanolamine buffer (pH 9.3) as the substrate. The color developed was read at 405 nm in a Multiscan (Flow Laboratories, Inglewood, Calif.) and compared with a control serum mixed with buffer. The percent inhibition was calculated as follows:

Percent inhibition = (1-(OD control − OD sample)/OD control) × 100.

Results

Studies with IMIF

The antibody titer of the untreated serum was determined by IMIF. Antibodies were detected in 4/10 samples. The untreated serum was then examined in parallel with serum previously treated with low pH and subsequently ultrafiltered. All 6 serum samples that previously had no detectable antibodies became positive after the low pH treatment. The antibody levels by IMIF in the treated serum ranged from 1:8 to 1:128. Furthermore, 2 serum samples that previously had detectable antibody prior to low pH treatment and ultrafiltration had an increased antibody titer. Anti-FA antibody levels in 1 serum remained unchanged whereas in the other, it dropped by one dilution after treatment. (Fig. 1 A).

In order to define the antibody specificity, we designed inhibition experiments to utilize antigenically pure FA. Incubation with purified FA resulted in the reduction of immunofluorescence in 7 of 10 serum samples (Fig. 1 B). In all but 1 of these serum samples, there remained detectable

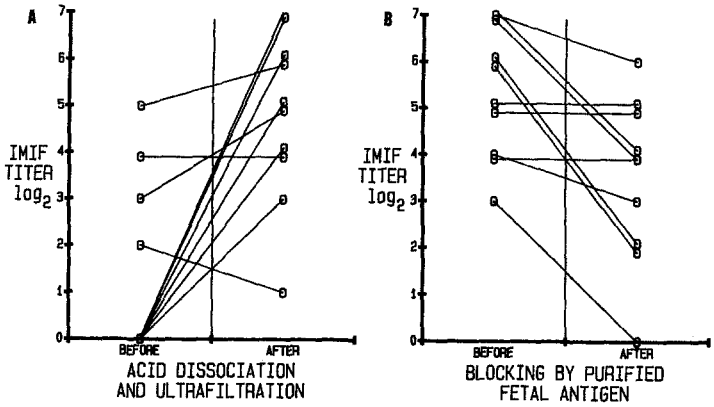

Fig. 1. Antibody titer in indirect membrane immunofluorescence (IMIF). Serum was initially treated with 0.1 *M* glycine HCl buffer (pH 3.1) and ultrafiltered through a 100×10^3 dalton exclusion limit membrane. The material retained on the membrane was collected and analyzed in IMIF. Panel A indicates the change in antibody titer following low pH treatment. Panel B demonstrated reduction in antibody titer following inhibition with purified fetal antigen (FA)

antibody following inhibition with purified FA suggesting the presence of antibody unrelated to FA in these serum samples. Quantitative inhibition experiments were then performed to confirm the nature of the antibody(s). In this series of experiments, the concentrated ultrafiltered material which presumably contained the antigen(s) (Fig. 2) and purified FA (Fig. 3) was used. The antigen fraction quantitatively inhibited immunofluorescence as did the purified FA. These results confirm that the serum from melanoma patients contained anti-FA antibodies and at least some of these antibodies were complexed, presumably in the form of IC to an immunologically similar FA.

Studies with ELISA

A competitive ELISA was utilized to detect FA in the filtrate of serum treated by low pH and ultrafiltered through a 100×10^3 dalton exclusion limit membrane. Because of the heat stability of FA [9] the filtered material was placed in a boiling water bath for 3 min prior to use in a competitive ELISA as described in *Materials and methods.* This resulted in the coagulation of the majority of serum proteins including albumin. The antigen(s) from the coagulated material was extracted with PBS. The extracts from the coagulated material, presumably antigen(s), exhibited quantitative inhibition of the binding of anti-FA antibody in the 7 serum samples that were known to contain anti-FA antibody in IMIF establishing that it contained an immunologically identical antigen (Fig. 4).

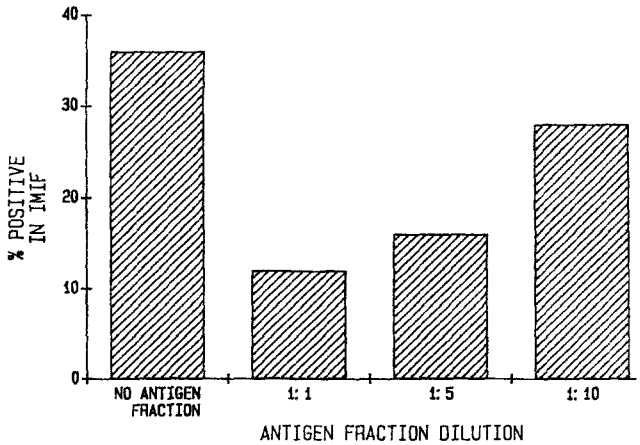

Fig. 2. Quantitative inhibition of antibody by the ultrafiltered material in IMIF. The ultrafiltered material was concentrated 6-fold on a YM membrane prior to analysis

Fig. 3. Quantitative inhibition of the low pH-treated antibody with purified FA (17.5 μg protein) in IMIF

Discussion

It is apparent that the antigenic composition of human melanoma is complex and highly heterogeneous and that some of these antigens are immunogenic in patients with melanoma. It would not be unreasonable to expect that this would result in the formation of CIC. In this investigation, serum from melanoma patients was exposed to an acid buffer to dissociate antigen/antibody complexes (CIC) and was studied by IMIF and in a competitive ELISA to determine if the IC contained FA and corresponding antibodies. The availability of a well-characterized and purified FA greatly simplified our task. Our results demonstrated that (1) a 69.5×10^3 dalton glycoprotein FA was present on the cell surface of culture melanoma cells, and

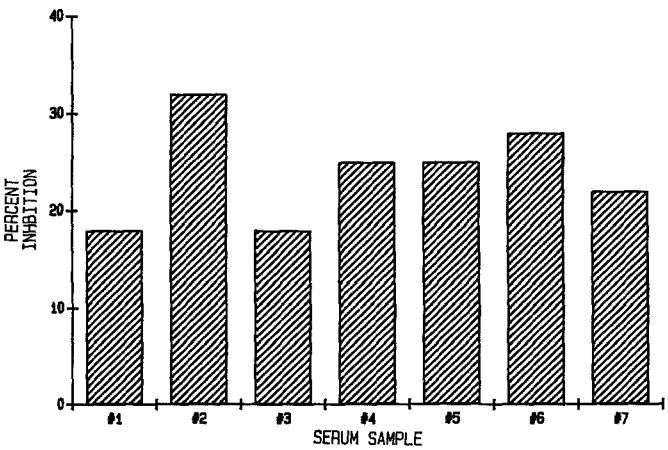

Fig. 4. Inhibition of allogeneic anti-FA antibody by the antigen fraction as determined by competitive inhibition in ELISA. Greater than 10% inhibition was considered positive for the presence of FA. The seven serum samples are those in which the augmented antibody response following low pH treatment was blocked by purified FA

(2) at least some of the IC of melanoma patients consisted of FA and corresponding antibody.

A number of antigens have been identified in IC of cancer patients, including TAA [4, 8, 20], oncofetal antigen [8, 26], viral antigens associated with virus-related malignancies [17, 29], idiotype/antiidiotype antigens [14], and altered self components [2, 5, 19]. It is generally agreed that TAA present in CIC of cancer patients comprise only a small fraction of the antigens complexed to antibodies [6]. Demonstration of the putative antigen and its relationship to disease has been achieved in certain malignancies including melanoma [31] and carcinoma of the lung [4].

Because of the high incidence of antibody to FA in serum from melanoma patients, we presumed FA to be immunogenic and this would result in the formation of CIC. The release of FA may be the result of a number of mechanisms. These include surface shedding, sublethal autolysis, and secretion [24]. Spontaneous shedding of TAA in the culture medium of tumor cells is well-documented [1, 7, 10].

A number of methods have been utilized to study the antigen composition of CIC. Acid dissociation and ultrafiltration was originally utilized by Sjogren et al. [30] to study lymphocyte cytotoxicity and more recently by Kirkwood and Vlock [14] to study autologous antibodies in CIC. This method appeared ideal to study FA because of its known physicochemical properties and the ability to prevent reassociation of the IC once the dissociating agent had been removed. Our initial results were consistent with those of Kirkwood and Vlock [14]. Pretreatment of serum with an acid buffer and ultrafiltration to prevent the reassociation of antigen/antibody complexes augmented antibody titers.

The specificity of the majority of antibodies remained to be determined. However, it is clear from the blocking experiments with purified FA that some of the antibodies present in the serum of melanoma patients were anti-FA antibodies. To confirm that FA was associated with these anti-FA antibodies, we investigated the ultrafiltrate which presumably contained the antigen. Our results with the antigen fraction demonstrated that an antigen immunologically similar to FA was present in the ultrafiltrate of sera of those patients with anti-FA antibodies.

Membrane immunofluorescence provides direct evidence for the cell surface location of the antigen. This is critical since only those antigens which are located on the cell surface might be accessible to antibody generated in active specific immunotherapy as well as being potentially important in the immunobiology of the tumor. However, this methodology is cumbersome and lacks sensitivity. For this reason, we undertook the development of an ELISA assay that could detect FA in the antigen fraction of the ultrafiltrate. Our results demonstated the feasibility of this approach.

The ability to detect a TAA in a sensitive immunologic assay provides potentially useful methodology to investigate antigen-specific CIC in cancer patients. The experimental design utilized in this investigation provides direct evidence that a 69.5×10^3 dalton glycoprotein antigen (FA) is present in the serum of melanoma patients in the form of CIC. Because melanoma is antigenically heterogeneous, it is expected that the availability of additional well-defined and well-characterized antigens will allow further characterization of the antigen makeup of CIC of cancer patients and the potential clinical utility will subsequently improve.

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Received February 26, 1988/Accepted May 2, 1988