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Isolation and Electrophoretic Analysis of Immune Complexes from Patients with Breast Cancer*

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Abstract—*Immune complexes (IC) were isolated from sera of patients with breast cancer (BC), from effusions from patients with breast and ovarian cancer (OC) and from effusions from patients with non-malignant diseases. Isolation was carried out with the aid of tubes coated with rheumatoid factor (RF) as immunoadsorbents. The effusions were first filtered through Sephacryl S-300 columns and the large molecular weight material eluted in front of the IgG peak was used as the starting material, while most of the sera were processed without prior filtration. The IC eluted from the RF-coated tubes were radioiodinated and analyzed by electrophoresis on polyacrylamide gels, followed by autoradiography. Four putative IC-derived proteins were identified in the RF eluates and designated according to their molecular weights (in Kdalton): p94, p46, p17 and p13. p94 was found in 1/8 BC effusions, but in none of the other samples tested; "p46", which was actually a complex of several closely spaced proteins, was identified in at least 3/16 BC sera, 6/8 BC effusions, 3/4 OC effusions and 1/5 non-malignant effusions, but in none of 17 normal sera. The upper band of the complex seemed to be present mainly, if not exclusively, in effusions from patients with neoplasia. p17 and p13 were found more often and in higher concentrations in the malignant effusions. All of these proteins could be precipitated by treating the RF eluates with anti human Ig serum, suggesting that they were associated with Ig, presumably as immune complexes.*

INTRODUCTION

THE PRESENCE of immune complexes in patients with malignant diseases has attracted considerable attention in recent years. Numerous studies have been carried out, largely with the object of investigating possible correlations between IC levels and the clinical course of the disease. In the course of these studies IC have been demonstrated in a large variety of neoplastic conditions (reviewed in ref [1]), including breast and ovarian cancer (BC, OC [2–12]).

In contrast to the extensive efforts directed at the identification of IC in malignancy, little work has been done so far on the much more difficult, though no less important, problem of the nature of the antigens involved in the formation of these IC. It is not unreasonable to assume that at least some of the IC in patients with cancer might consist of tumor-associated antigens, and preliminary work in this area appears to confirm this assumption [10, 13]. It would be extremely important to establish this point conclusively. The identification of tumor-associated antigens in IC might open new avenues of research in human tumor immunology, not the least of which would be the

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development of new reagents for immunodiagnosis.

In continuation of our earlier studies [13], we have used a technique recently developed in our laboratory [14, 15] to isolate and analyze IC from sera and effusions of patients with BC. Similar preparations isolated from normal sera and from effusions of patients with OC or with non-neoplastic diseases were analyzed as controls. The results are presented below.

MATERIALS AND METHODS

Reagents

Blood samples were obtained from unselected BC patients and from healthy blood bank donors. The blood was left to clot for 3–4 hr at room temperature, after which sera were separated and stored at −70°C. Effusions removed therapeutically from patients with advanced disease were stored at −20°C. Sera with high titer Rheumatoid Factor activity were obtained from patients with rheumatoid arthritis and stored at 4°C with 0.1% sodium azide. Antisera to bovine and human serum albumin (BSA and HSA respectively) and to human IgG and IgM were prepared in rabbits by injecting the antigens emulsified in complete Freund's adjuvant, according to standard procedures. Anti actin and polyvalent anti HLA sera were gifts from Drs. Y. Gazitt and H. Brautbar, respectively, of this department. Anti β_{2m} serum was obtained commercially. Ig fractions of the antisera were prepared by precipitation with ammonium sulfate, at 33% saturation. Borate Buffered Saline (BBS), pH 8.0, was used as diluent or for washing tubes and precipitates.

Assay for IC

Sera were tested for IC content by a slight modification (Sulitzeanu *et al.*, in press) of the complement consumption assay[16]. BSA–anti BSA immune complexes added to normal human serum were used as standards for IC determinations. Sera were selected for further study if their anti-complementary activity was at least as high as that of the standard serum containing 15 μg BSA and 25 μg anti BSA antibody per ml (i.e., 3× antigen excess). Effusions were processed without preliminary IC determination.

Gel filtration

The IC content of most of the sera was rather low, while that of the effusions was below detectable levels. To increase the IC concentration, sera and effusions were concentrated 2 to 3-fold by filtration through Centriflo cones (Amicon, U.S.A.), according to the manufacturer's directions. All the effusions and a few of the sera were subsequently filtered through Sephacryl columns (S-200 in the initial and S-300 in the later experiments, 2 × 120 cm and 3 × 105 cm respectively) at a rate of 6 ml/hr. The eluent contained 0.5 M NaCl, 6 mM sodium borate, pH 7.4, and 0.02% sodium azide. The first peak eluted was concentrated by Centriflo filtration to a small volume (1–3 ml) and dialyzed against isotonic borate buffered saline.

Isolation of IC by adsorption to RF coated tubes

Details of the technique have been published [14, 15]. In brief, polystyrene tubes coated with IgG were treated with RF serum at 4°C in a tissue culture rotator. The RF was used either as whole serum or after partial purification, as follows: a pool of RF sera was diluted 10-fold in water, treated with EDTA (final concentration 0.02 M) and dialyzed for 48 hr against 3 liters of water, pH 7, with 2 changes. The resulting precipitate was dissolved in BBS and sedimented at 6000 *g* for 10 min to remove insoluble material.

The RF-coated tubes were washed 3 times, IC-containing sera or the first Sephacryl peak fractions (0.5 ml) were added and the tubes rotated for 24–48 hr at 4°C. The contents of each tube were washed 3 times and the bound complexes eluted with 0.3 ml of 0.1% SDS in phosphate-buffered saline (PBS) by rotating the tube for 2 hr at 37°C. As shown previously in experiments with model IC [14, 15], this procedure resulted in the elution of over 90% of the bound antigen. In later experiments, sera or Sephacryl fractions were treated with EDTA (0.02 M final concentration, 15 min at 25°C) before adsorption to RF tubes, to remove complement components. The capacity of RF tubes to bind IC was checked with the aid of radioiodinated BSA–anti BSA IC, prepared in human serum [15]. Briefly, 15 μg BSA, containing a trace amount of labelled BSA and 25 μg anti BSA antibodies, in a volume of 25–50 μl, were added to 1 ml NHS and the mixture was incubated with the RF-coated tubes for 2 days. The radioactivity bound was then determined. RF was considered as satisfactory if at least 5% of the radioactivity remained bound to the tube.

Labelling of isolated IC

Isolated IC preparations of 0.3–0.6 ml (depending on whether one or 2 RF tubes were used) were iodinated with 0.3–0.5 mCi of $Na^{125}I$ (Amersham, England). After iodination,

400 μg of "carrier" BSA and 2 mM phenylmethyl sulfonyl fluoride (PMSF) were added and the non-bound ^{125}I was removed by extensive dialysis against borate-buffered saline containing 0.5 mM PMSF. Efficiency of labelling was 10–20% of the ^{125}I added.

Purification of radioiodinated IC

It became evident during the initial experiments performed in this study that IC recovered from the RF tubes were contaminated with serum proteins, mainly HSA and immunoglobulins (the latter derived, at least in part, from the RF and the IC themselves). In the experiments carried out with the effusions, the contaminants were removed by treating the samples with anti HSA and anti Ig sera, after first dissociating the IC at low pH, as follows: to the IC preparation, 1/3 volume of 1 M glycine–HCl buffer, pH 2.3, was added and the mixture was kept at 4°C for 20 min. After neutralizing with 0.5 M Tris (Trizma Base, Sigma, U.S.A.) one of the following procedures was adopted: (a) coprecipitation: 5–10 μg of each antigen (HSA, etc.) was added, followed by an equivalent amount of the corresponding antiserum (Ig fraction). The mixtures were incubated overnight at 4°C and the precipitates were removed by centrifugation; (b) treatment with *Staphylococcus aureus* Cowan I strain [17]: Ig fractions of the antisera (50 μg of each) were added to the IC samples and the mixtures were incubated as before. Sufficient bacteria were then added to bind the total Ig present, the mixtures were incubated for 15 min at room temperature and then centrifuged.

SDS-PAGE and autoradiography

The labelled IC preparations were analyzed by SDS-PAGE, according to the procedures of Studier [18] and Laemmli [19], in the Hoeffer Slab gel electrophoresis apparatus. Gradient gels of 8–21% polyacrylamide with 0.1% SDS were 1.5 mm thick and 270 mm long. A stacking gel of 5% polyacrylamide, containing 10 or 20 sample wells, was cast above the separating gel. Each well was loaded with proteins containing equal amounts of radioactivity. The proteins were prepared for electrophoresis by boiling for 3–6 min in a buffer containing 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.005% bromphenol blue and 50 mM Tris – HCl, pH 6.8. In some experiments the 2-mercaptoethanol was omitted in order to analyze non-reduced proteins. Electrophoresis was performed at 10 mA for 16–18 hr. The gel was fixed, stained and dried for autoradiography. Protein bands were visualized by autoradiography using Kodak's X-omat MA or AGFA's Curix RP2 X-ray film, and a Quanta II intensifying screen (DuPont). Molecular weight of the proteins was estimated from standard curves constructed using HSA, ovalbumin, and cytochrome C as markers.

RESULTS

General approach

The approach followed in this investigation was as follows: immune complex-like materials were isolated from sera and effusions, were labelled with ^{125}I and were analyzed by SDS-PAGE, followed by autoradiography. Protein bands found exclusively or mainly in samples obtained from patients with malignancy were assumed to be tumor markers or tumor-associated antigens derived from immune complexes.

Isolation and analysis of IC were performed by a technique developed as a result of extensive work carried out with a variety of model antigen–antibody systems: tetanus toxoid–human anti toxoid antibodies; BSA–anti BSA; ovalbumin–anti ovalbumin [14, 15]. It was found in this work that tubes coated with RF will bind a small amount of IC with a sufficient degree of purity to enable the antigen portion of the complexes to be identified unambiguously.

It became clear during the initial stages of this work that contamination of IC with non-specifically bound proteins was a major problem. The following steps were therefore taken to reduce contamination: (a) samples were treated with EDTA to remove, as far as possible, complement components; (b) samples were fractionated on Sephacryl columns before applying them to the RF-coated tubes. This yielded more concentrated IC preparations, which, being also largely free of non-complexed IgG, could bind to RF more efficiently, due to minimal competition from IgG; (c) radioiodinated IC were treated with anti Ig and anti HSA sera to remove Ig and HSA, which were the major contaminants, thus resulting in much "cleaner" autoradiographic patterns.

Finally, the relationship of the identified antigens to IC was proved by coprecipitation experiments, which showed that the antigens coprecipitated with IgG on treatment with anti IgG serum, but failed to do so if the samples were first treated with low pH buffer in order to dissociate the IC.

Analysis of IC isolated from sera

Isolation of IC from sera was performed, in most cases, by reacting directly the unfrac-

tionated serum with RF-coated tubes, but a few analyses were also carried out with Sephacryl-fractionated sera. IC isolated from 16 BC sera were compared to identical preparations obtained from sera of 17 healthy blood bank donors. Five of the sera in the latter group were selected on the basis of their high content of "IC-like" activity.

The autoradiographic patterns were quite complex, the predominant bands being produced by the immunoglobulins [IgM (RF), IgA and IgG] and human serum albumin. Most of the bands in the BC sera seemed to have counterparts in the normal sera. However, two molecular species were distinguished which appeared to be present only in, or in significantly elevated levels in, the BC samples: a protein of 46 Kdalton (p46, Fig. 1, lanes 4, 7, 10) was present as a broad band in 3 of the 16 BC sera, but appeared to be absent or present in markedly reduced levels in the 17 normal sera tested. p46 was found in both non-reduced (Fig. 1) and reduced (Fig. 2) samples, and was recovered both from non-fractionated and from the first peak of Sephacryl-fractionated sera. Thus, this protein appeared to be associated with a large molecular weight complex, but the attachment was non-covalent, since it could be readily dissociated by SDS in the absence of reducing agent; a second protein, of 17 Kdalton (p17), was identified in 5 of the BC sera, but there was a similar, though much fainter band in some of the normal sera. This molecule appears to be covalently bound to a higher molecular weight protein, since it was only found in preparations reduced with 2-ME. The difference in the intensity of the 17 Kdalton band between the normal and the BC sera most probably reflected concentration differences, since the total radioactivity placed in each lane was constant for any given gel. p17 was found independently of the p46 protein, e.g. a sample might contain one but not the other.

Analysis of IC isolated from effusions

All analyses in this series were performed with the modified technique: the IC were first enriched by Sephacryl filtration and, after radioiodination, were freed of contaminating proteins by treatment with the antisera, as described in Materials and Methods. The material tested included 8 effusions from patients with BC, 4 from patients with OC and 5 from patients with non-malignant diseases. Figure 3 shows the electrophoretic patterns obtained with 3 effusions, 2 from patients with BC and 1 from a patient with heart insufficiency. p46 and p17 are again seen as prominent bands in the BC effusions, but are much fainter in the non-malignant preparation. A new protein of 13 Kdalton molecular weight (p13) was also identified in this and subsequent experiments. It also appeared to be more concentrated in the BC samples. p13 was found in 4/8 BC effusions and in 1/4 OC effusions (see below), but was seen only as traces in the non-neoplastic effusions.

To determine whether these proteins were complexed with antibody, the IC were precipitated with anti Ig sera, with or without prior dissociation at low pH (Fig. 4). When the precipitation was carried out after low pH treatment, the putative IC antigens p46, 17 and 13 remained in the supernate. When precipitation was performed using non-dissociated samples, however, these proteins were found in the precipitate, strongly suggesting that they were associated with Ig, presumably antibody.

One of the IC preparations isolated from a BC effusion contained an additional protein, of 94 Kdalton molecular weight (p94), identified as a heavy band in non-reduced gels (Fig. 5). This protein, which was not seen in any of the other samples, was also associated with Ig, as it could be coprecipitated with the Ig–anti Ig system.

Figure 6 shows results of analyses of 4 effusions from patients with BC, 4 from patients with OC and 1 from a patient with liver cirrhosis. p46 and p17 are present in 3/4 OC samples, while p13 is seen in 1/4. However, it is evident that p46 actually consists of more than one protein (lanes 3, 4 and 8), as shown in greater detail in Fig. 7. Four different bands can be distinguished in the p46 region, of which the uppermost one is quite strong in some of the BC and OC effusions. This band was very faint or totally absent in the non-malignant effusions.

The distribution of the various proteins identified in these analyses is summarized in Table 1.

It has been reported recently [20] that antigens of several human neoplasms, including BC, are associated with β_{2m}. To determine whether any of the IC-derived proteins were likewise associated with β_{2m}, radioiodinated RF eluates were treated, after acid dissociation, with either anti HLA or anti β_{2m} serum, the mixtures were treated with Staphylococci and the radioactivity bound was analyzed in gels. None of the IC proteins could be precipitated by this procedure. Similar experiments, performed with antiactin antiserum in order to

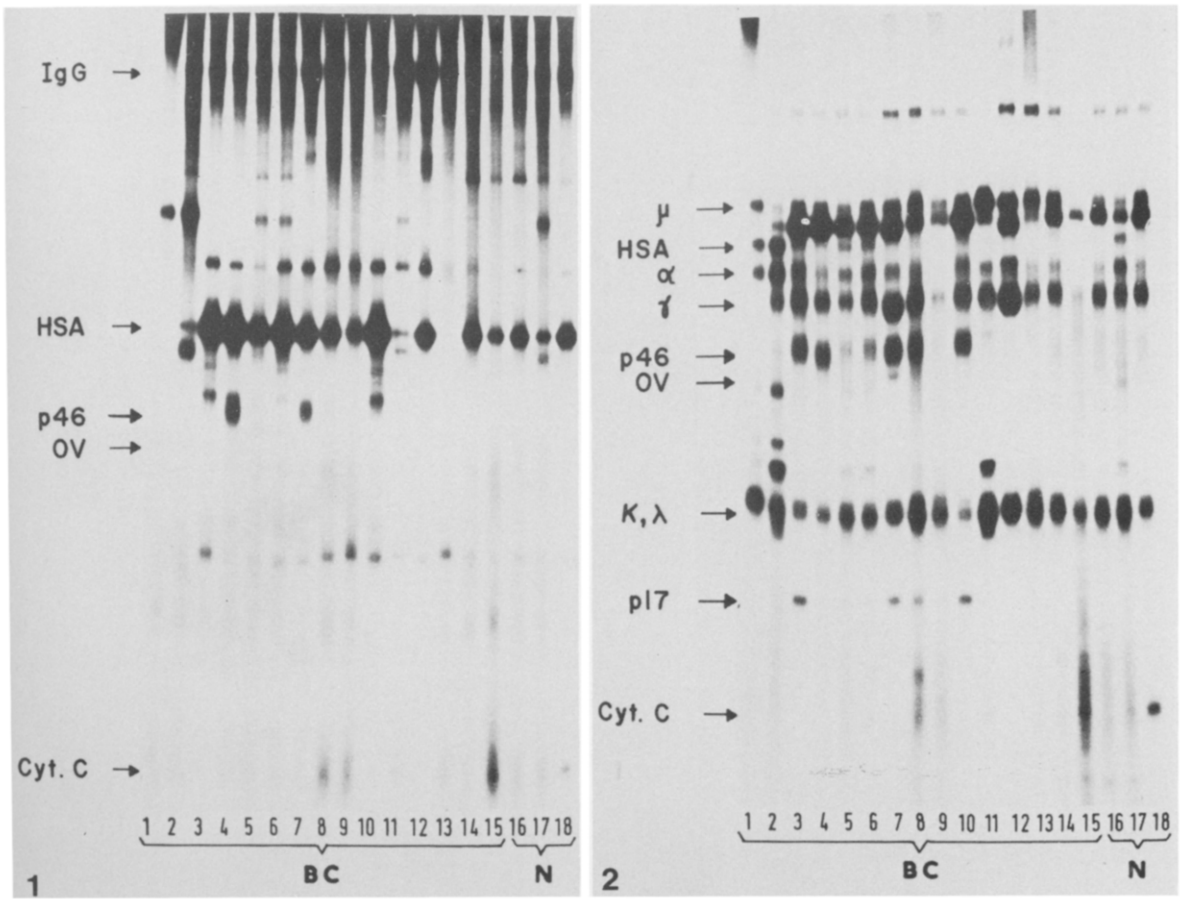
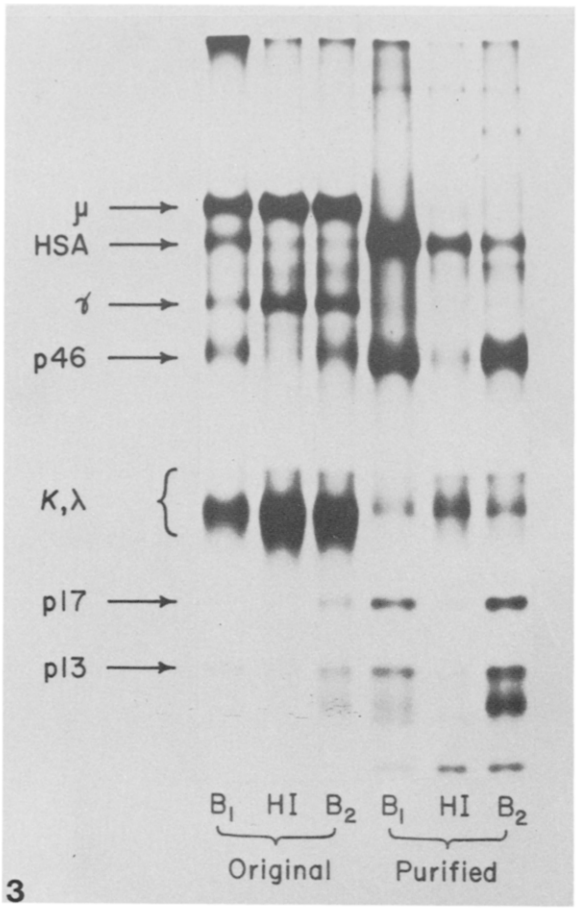


Fig. 1. *SDS-PAGE analysis of IC isolated from BC (lanes* 1–15) *and normal (lanes* 16–18) *sera: non-reduced samples.*

Fig. 2. *SDS-PAGE analysis of IC isolated from BC (lanes* 1–15) *and normal (lanes* 16–18) *sera: samples reduced with* 2-*ME.*



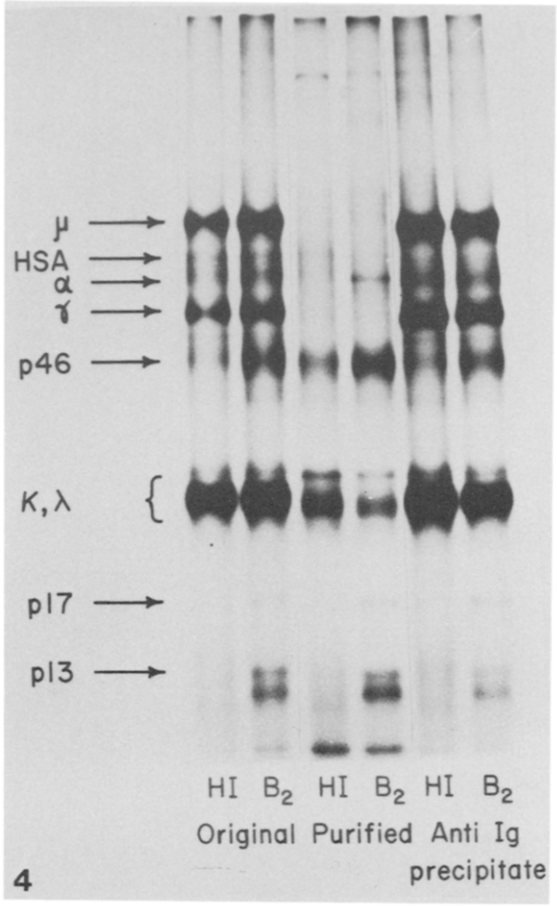


Fig. 3. *SDS-PAGE analysis of IC isolated from effusions.* B_1, B_2: *patients with breast cancer*; *HI*: *patient with heart insufficiency*; *original*: *original RF eluates*; *purified*: *RF eluates purified by treatment with anti IgG and anti IgM serum, followed by Staphylococci. Anti HSA serum was not used in this experiment*; μ, γ: *heavy Ig chains*; κ, λ: *light chains. Samples were reduced with 2-mercaptoethanol.*

Fig. 4. *Precipitation of IC with anti Ig serum. IC isolated from patient with heart insufficiency* (*HI*) *and breast cancer* (B_2). *Original*: *original RF eluates*; *purified*: *supernates of RF eluates dissociated at low pH, then treated with anti HSA and anti Ig sera. The putative IC antigens* (*p*46, *p*17 *and p*13) *were not removed*; *anti Ig precipitates*: *precipitates of non-dissociated RF eluates coprecipitated with anti IgG antibodies. The putative IC antigens are found in the precipitates. Samples were reduced with 2-mercaptoethanol.*

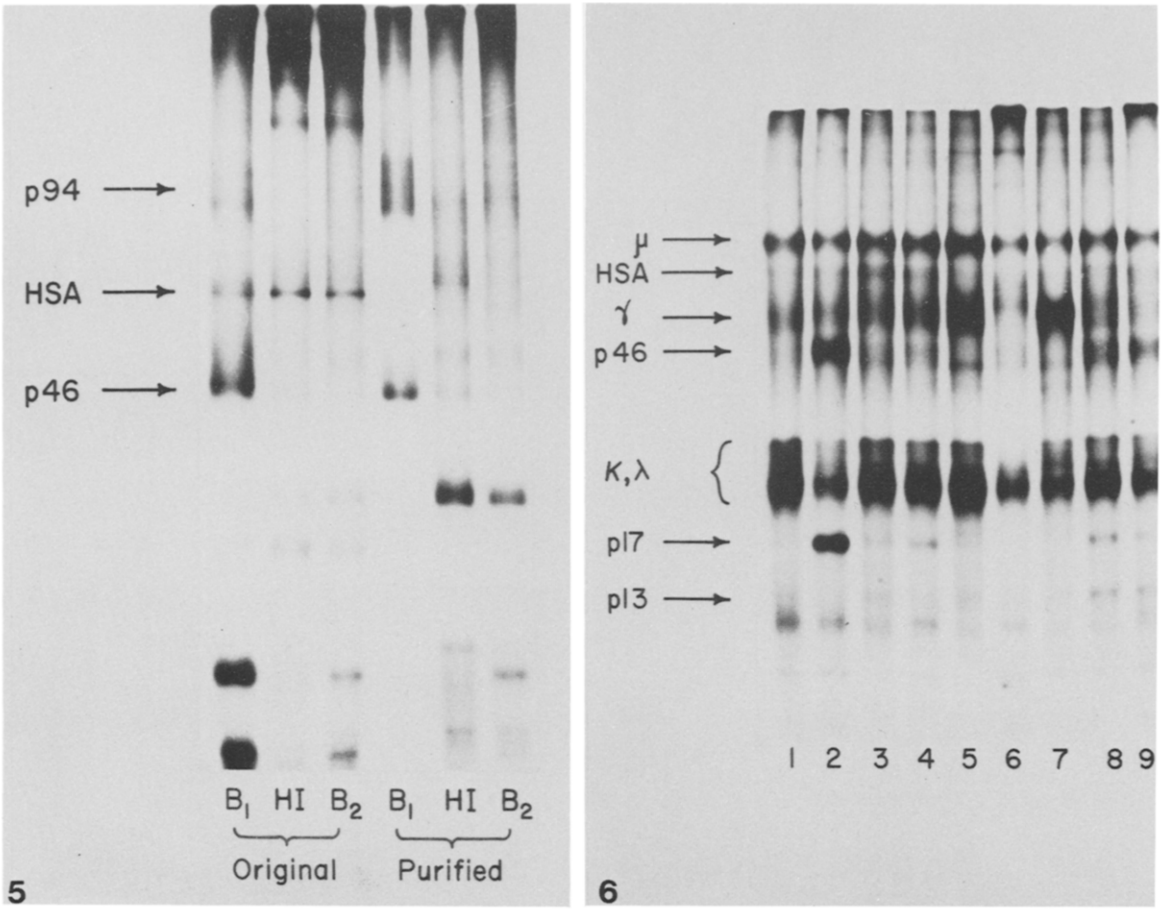


Fig. 5. SDS-PAGE analysis of non-reduced IC isolated from effusions. B_1, B_2:: Patients with breast cancer; HI: patient with heart insufficiency; original: original RF eluates; purified: samples purified by treatment with antisera.

Fig. 6. SDS-PAGE analysis of non-purified IC from effusions. Lanes 1, 2, 4, 9: ovarian cancer; 3, 6, 7, 8: breast cancer; 5: liver cirrhosis. Samples reduced with mercaptoethanol.

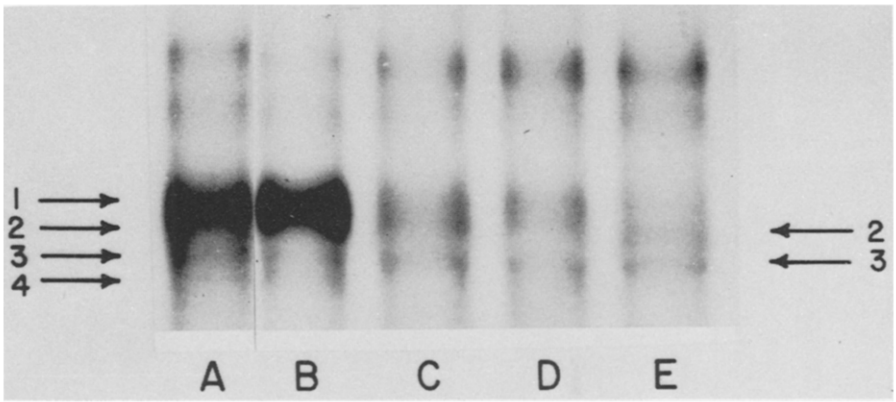


Fig. 7. "p46" complex, showing the 4 bands distinguishable in the complex. The upper band (No. 1) is practically invisible in lane E (effusion from patient with liver cirrhosis). A, B, D: effusions from patients with ovarian cancer; C: effusion from patient with breast cancer.

Table 1. Proteins identified in RF eluates from malignant and non-malignant effusions

Source of effusions	Proteins 94	46 S*	46 T†	17 S	17 T	13 S	13 T
Breast cancer	1/8	6/8	0/8	6/8	0/8	4/8	0/8
Ovarian cancer	0/4	3/4	0/4	3/4	1/4	1/4	0/4
Non-malignant‡	0/5	1/5	4/5	0/5	4/5	0/5	2/5

*S = Strong band.
†T = Weak band or trace.
‡Effusions from: patients with heart insufficiency (2); liver cirrhosis (2); and not-diagnosed disease (1).

examine the possibility that p46 might be identical to actin, proved also to be negative.

DISCUSSION

The presence of IC in sera of patients with cancer is well established [1] and some evidence has also been produced for their presence in effusions of patients with BC [10, 13]. However, very little work has been done so far on the identity of the antigens in IC, largely because of the great difficulties encountered in isolating the tiny amounts of IC usually available.

In an earlier extensive search for IC in effusions from patients with BC, we found 4 positive samples among the 23 samples tested [4]. A protein of approximately 70 Kdalton, partially purified from IC isolated from one of the effusions, reacted preferentially with sera of patients with BC [13], but its extreme lability precluded more intensive study. Recently, Papsidero *et al.* [10] isolated from IC, derived from a pleural effusion of a patient with adenocarcinoma of the breast, an antigen of similar size (67 Kdalton), as well as 3 additional antigens. The molecular weight of these species was not determined. The antigens were assayed by their ability to interact with IgG purified from the same pleural fluid.

In our experiments, IC were isolated using tubes coated with RF as immunoadsorbents. This technique was developed with the help of artificial IC prepared with tetanus toxoid–human anti tetanus antibodies [14] and with BSA–rabbit anti BSA antibodies [15]. The experiments with these model systems confirmed that the RF tubes bound the antigens only when the latter were complexed with antibodies, and that binding was proportional to the concentration of IC. In further studies, the RF technique was employed to isolate IC from sera of patients with Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC). The isolated IC were subjected to SDS-PAGE and tested for the presence of glycoprotein antigens by treating the gels with radioiodinated concanavalin A. Two new proteins, identified as a double band of 40 Kdalton molecular weight, were seen in 80% of the BL and NPC sera, but in none of the 30 control sera obtained from healthy donors or from patients with a variety of neoplastic diseases [15]. It was thus established that the RF technique could be employed to analyze IC in clinical samples.

SDS-PAGE analyses of IC isolated from sera of patients with BC revealed the presence of a broad band of approximately 46 Kdalton molecular weight ("p46") in at least 3 of the BC sera but in none of the 17 normal sera. An identically-migrating protein set was found in 6/8 BC and 3/4 OC effusions and, possibly, in one of the non-malignant effusions. In the remaining non-malignant effusions only a weak band was seen at the p46 position. p46 appeared to consist of 4 separate, but closely spaced, proteins. In a few gels, in which the 4 bands could be distinguished, the upper and heavier band was only observed in the BC and OC effusions. It thus seems likely, although this is by no means certain, that the upper band was associated mainly, if not exclusively, with neoplasia. There is little doubt, on the other hand, that the concentration of p46 complex was considerably higher in effusions from patients with neoplasia.

Two additional proteins, p17 and p13, were found consistently in higher concentrations in RF eluates from BC and OC effusions. Only the former was identifiable in preparations isolated from sera. p17 was seen exclusively in samples reduced with 2-ME, suggesting that it was a subunit of a larger protein. p13, on the other hand, could be detected in both reduced and non-reduced gels.

One of the BC effusions contained yet another protein, of 94 Kdalton molecular weight, visible as a broad band on the autoradiogram. As it was only found in one sample, its true nature remains uncertain, but it is remarkable that a protein of similar or identical size has been detected recently in a wide variety of neoplastic cells [21–24].

It could be argued that the proteins found in the eluates from the RF tubes might not be true IC components, but simply substances non-specifically bound to the tubes. Two findings render this possibility unlikely. First, all the putative IC proteins were recovered from the large molecular weight fraction (> 150 Kdalton) eluted from Sephacryl. Since, with the possible exception of p17, they are all small molecules (as shown by their migration in gels

under non-reducing conditions), they must have eluted from the column as large molecular weight complexes. Second, the putative IC proteins could be precipitated with anti Ig antibodies, but remained largely in the supernate if the eluates were first dissociated with low pH buffer. It appears reasonable, therefore, that they were complexed with Ig, presumably as immune complexes. Attempts to reassociate the complexes failed, however, possibly because of the denaturation of the molecules resulting from the SDS treatment during the elution procedure, and/or because of their small concentrations.

The antigenic make-up of the BC cell has been studied extensively in recent years. Using the blocking tube leukocyte adherence inhibition test to monitor their fractionation experiments, Thomson and his co-workers [20, 25–27] have purified from BC tissues a 70–150 Kdalton antigen associated with β_{2m}. On SDS-PAGE, this antigen was dissociated into 3 fragments of 40, 25 and 12 Kdalton molecular weight. Similar results were obtained with material purified from urine [27]. Leung and collaborators [28–30] have isolated, with the aid of a xenogeneic antiserum, a 53 Kdalton and a smaller, 20 Kdalton molecule, which are apparently specific for BC. Whether these molecules are related to the antigen reported by Ohno *et al.* [31], which was identified by its cross-reactivity with gp52 of MMTV, is not known. Holton *et al.* [32] employed antibodies specifically purified from sera of patients to isolate from BC cells 2 molecules, of 69 and 19 Kdalton. With the possible exception of the 19 Kdalton antigen of Holton *et al.*, which is similar in size to the p17 molecule identified in our work, all BC-associated antigens enumerated above would seem to differ from the proteins identified in our IC studies. Unlike the 40 Kdalton protein of Thomson *et al.* [20], the proteins of the p46 complex did not seem to be associated with β_{2m}, since they could not be precipitated with β_{2m} antiserum. One cannot rule out the possibility that "p46" may be degradation products of larger molecules, or that the reactivity with the β_{2m} antiserum may have been lost as a result of the SDS treatment. It seems more likely, however, that, as suggested by Davis *et al.* [33], the BC cell might contain a multitude of tumor-associated antigens.

Most of the putative IC-derived proteins (with the exception of p94 and, possibly, the upper band of the p46 complex) were also found in the non-malignant effusions, albeit in smaller amounts. These are likely to be, therefore, auto-antigens, rather than tumor-associated antigens. Patients with BC are known to make auto-antibodies to several auto-antigens, including smooth muscle (short review in ref. [34]). It has been shown [35] that the smooth muscle auto-antibodies react with actin, a protein which has the same size as the "p46" complex. However, attempts to precipitate "p46" with antiactin antibodies failed, indicating that "p46" does not include actin.

In conclusion, using a technique known to isolate efficiently model immune complexes, we have identified four proteins in IC isolated from BC and OC effusions. The p94 molecule, which was found in one BC effusion, might be a BC-associated antigen. The upper band of the p46 complex, which was found in both BC and OC effusions, might be an antigen associated with two or several tumors. The p17 and p13 molecules may be tumor markers, e.g. normal proteins found in increased concentrations in patients with cancer. Final characterization of these proteins will be possible only after they are purified and antibodies against them become available. For the present, it can only be concluded that these proteins appear to be present in higher concentrations in patients with breast and ovarian cancer than in patients with non-malignant disease or in healthy individuals.

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