AN IMMUNE COMPLEX ASSAY FOR SV40 T ANTIGEN

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SUMMARY. An assay is described for the detection and quantitation of T antigen, a protein present in cells transformed or infected by simian virus 40. In principle it is a direct immunoprecipitation of the antigen with serum from tumor bearing hamsters, followed by collection of the immune complexes formed on glass fiber filters. The complexed immunoglobulin is then exposed to ^{125}I-labeled protein A, a protein with affinity for the Fc region of many immunoglobulins. The amount of protein A bound is then related to the amount of immunoglobulin and thus of antigen on the filter. With the appropriate sera this assay can probably be used for many other large antigens of biological interest.

DNA tumor viruses such as polyoma and simian virus 40 (SV40) induce a small number of proteins in the cells they infect. Of these the first to appear is T antigen, a virus-specific protein which also appears in transformed and tumor cells and is usually recognised by its reaction with sera from animals immunised with virus-free transformed cells (1, 2). Because of its role in the virus replication cycle and probably in the maintenance of the transformed state, it is important to characterize T antigen and, in particular, to elucidate its biochemical activity. To date the assays used for T antigen have all been immunological and detect the presence of the antigen plus any cross-reacting materials in cells. All the assays, immunofluorescence, immunoprecipitation and complement fixation, have limitations both with respect to quantitation and recognition of the reaction species (3).

The immune complex assay described here was developed in an attempt to circumvent these problems and provide a more convenient way of following T antigen during biochemical characterization. It makes use of two observations, firstly the trapping of immune complexes on glass fiber filters (4) and secondly the strong affinity of the protein A from Staphylococcus aureus for the Fc region of most classes of IgG (5).

Abbreviations: SDS, Sodium dodecyl sulfate

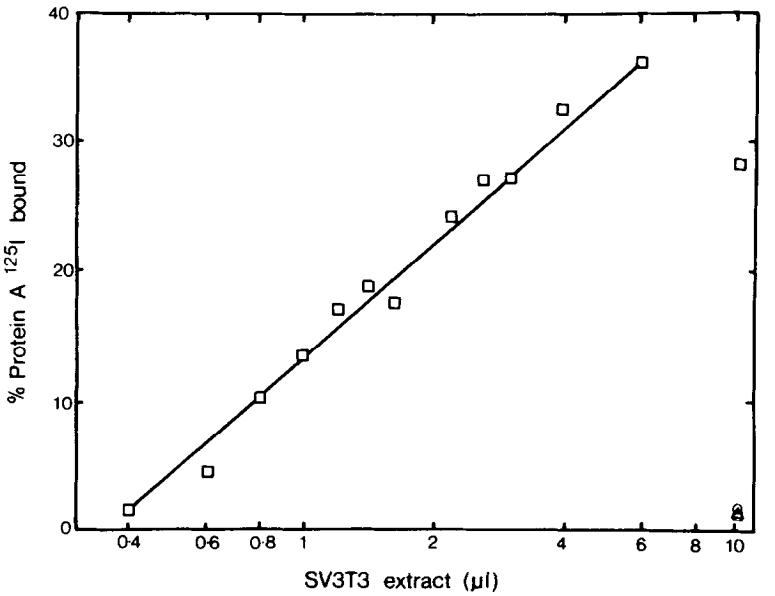

Fig. 1. The effect of varying antigen concentration. Parallel immunoprecipitations were set up containing from 0.4 to 10 µl of an extract of SV40 transformed mouse cells (SV3T3) and 7 µl of anti-T serum from hamsters bearing tumors induced by SV40 transformed hamster cells (SV28). Controls of transformed cell extract with normal hamster serum △, untransformed 3T3 cell extract with normal hamster serum ⊠, and with anti-T serum O, are also shown.

MATERIALS AND METHODS

Protein A. Protein A isolated from Staphylococcus aureus was obtained from Pharmacia Fine Chemicals, and iodinated using either the chloramine T method (6) or the Bolton and Hunter reagent (7). ^{125}I and the Bolton and Hunter reagent were both obtained from the Radiochemical Centre, Amersham.

Anti-T sera were obtained from hamsters bearing tumors induced by subcutaneous inoculation of 10^7 cells of an SV40 transformed hamster cell line (SV28). The anti-T sera and control sera from uninoculated animals were filtered (Whatman GF/C glass microfibre) before use.

Cell extracts were prepared by freezing and thawing 10^7 cells in 0.3 ml of extraction mixture (80 mM NaCl, 20 mM EDTA, 20 mM Tris pH 8.0, 1 mM DTT, 200 µg/ml phenyl methyl sulphonyl fluoride (Calbiochem) and 10% glycerol) and spinning off cell debris (10,000 g for 30 min).

Filter binding assay. Immune complexes were prepared by mixing cell extracts and sera in NET (8) (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4) containing 2 mg/ml bovine serum albumin 0.02% sodium azide and 0.05% NP40. Up to 25 µl of extract and 25 µl of serum plus 50 µl of the buffer were mixed and incubated for 20 hours at 4°C before loading on to filters. Filters (5 mm disks of GF/C glass microfibre, Whatman) held in 1 ml dis-

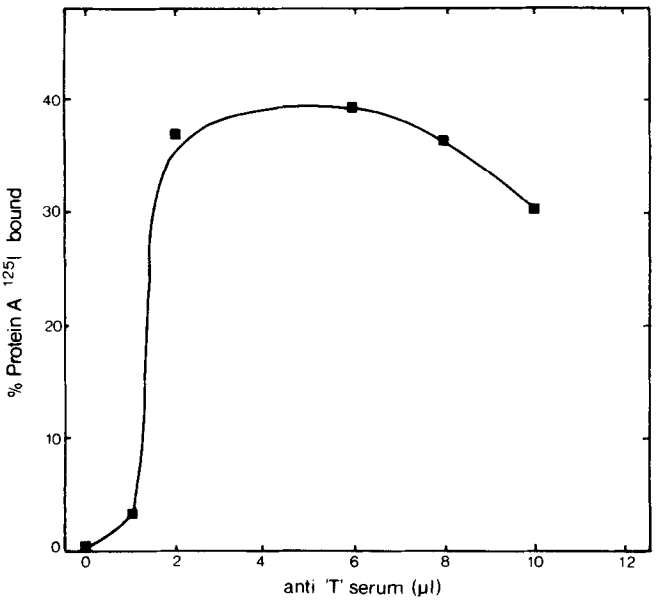

Fig. 2. The effect of varying antibody concentration. Parallel immunoprecipitations were set up with 20 µl of SV3T3 extract and 1 to 10 µl of hamster anti-T serum and treated as described above.

posable plastic syringe barrels were wetted with NET containing 0.25% gelatin and 0.05% NP40 before loading the samples and washing with 1 ml of the same buffer. After blowing through the excess liquid 10 µl of ^{125}I protein A (10^4 cpm, 10 ng) in buffer was pipetted directly on to the filter and allowed to soak in. All the filters in a set were then counted in a Mini-assay type 6-20 gamma counter allowing 5 to 10 min at room temperature for reaction of the protein A with the immune complexes on the filters before washing as above and counting again. The background of the counter was 100 to 150 cpm and blank filters incubated with serum alone or extract alone bound less than 1% of the added ^{125}I.

RESULTS

Quantitation of the assay. The effect of varying antigen concentration on protein A ^{125}I binding is shown in Figure 1. Parallel immunoprecipitations were set up containing from 0.4 to 10 µl of an extract of SV40 transformed mouse cells (SV3T3) and 7 µl of anti-T serum. It can be seen that the amount of protein A ^{125}I bound is proportional to the amount of antigen added on a logarithmic scale in the range 0.4 µl to 6 µl of this extract. For many purposes this is the most important characteristic of the assay. The sensi-

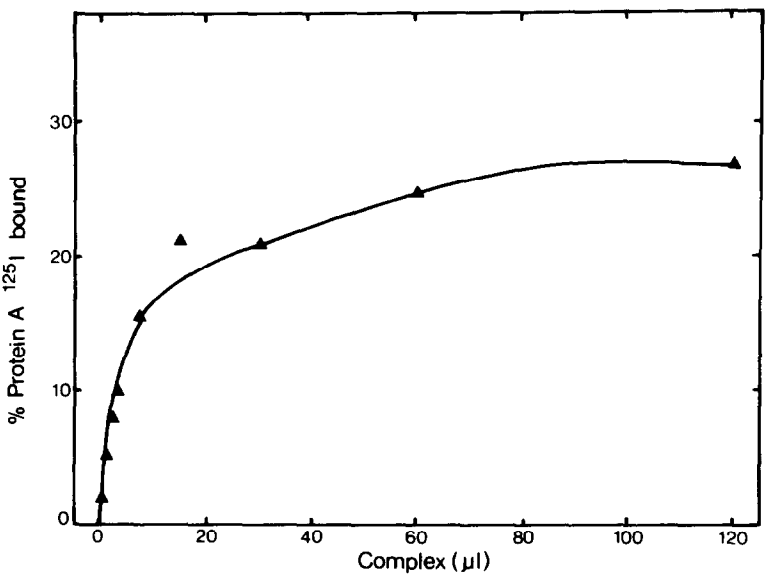

Fig. 3. The effect of varying input of preformed immune complex. A mixture of 40 µl of SV3T3 extract, 30 µl of anti-T serum and 230 µl of buffer was incubated for 20 hours at 4°C. The volumes shown were then loaded on to a series of filters and treated as described above.

tivity of the assay can be increased by prolonged incubation of the complexes. All control filters show negligible levels of protein A ^{125}I binding.

Figure 2 shows the effect of varying the amount of antibody, this demonstrates that for the usual assay of antigen, the antibody is in considerable excess. Assay of increasing amounts of complex (formed at a particular antigen and antibody level) shows that the amount of protein A ^{125}I bound increases rapidly at first and then approaches a plateau at high levels (Fig. 3).

Analysis of the filter binding material. To determine the composition of the immunoprecipitates retained on the filters it is convenient to use cell extracts labeled with ^{35}S methionine. The ^{35}S interferes very little with counting of the ^{125}I in a gamma counter. Parallel filters made with the same immunoprecipitates and washed in the same way as described above but omitting the ^{125}I protein A, were extracted with electrophoresis sample buffer containing sodium dodecyl sulphate at room temperature. This dissociates the immune complexes and after heating, the samples were ready for gel electrophoresis and fluorography (Fig. 4). It can be seen that the major band

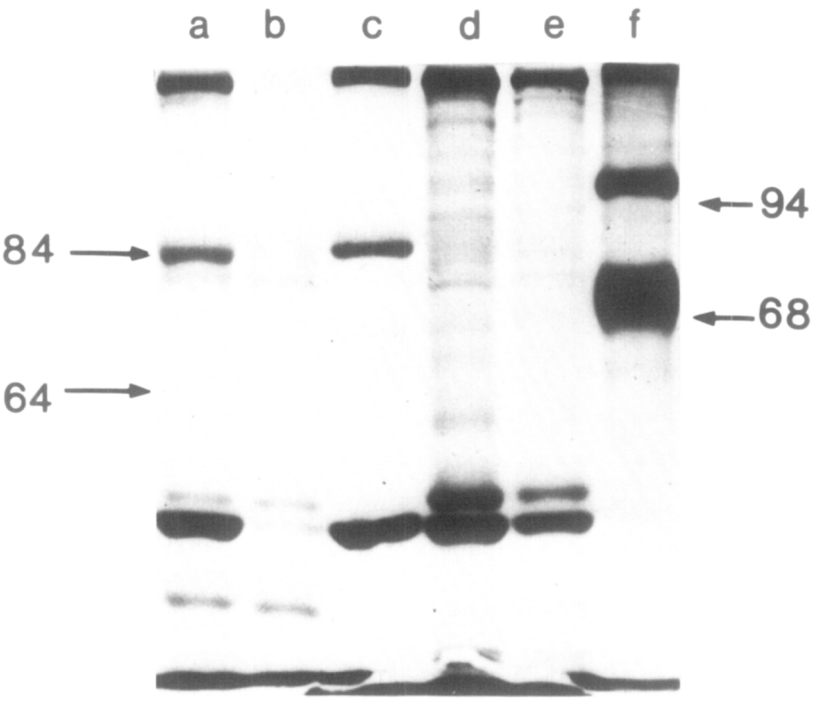

Fig. 4. Comparison of immunoprecipitates collected on filters and by adsorption to bacteria carrying protein A. Immunoprecipitates were prepared from an extract of SV40 infected monkey cells (CV1), labeled with ^{35}S methionine from 69 to 72 hours after infection using the same hamster anti-T serum as before (Fig. 1). Parallel incubations were adsorbed to protein A bacteria (8) and eluted with 2% sodium dodecyl sulfate (SDS) (a), or filtered through GF/C and the filtrate treated with protein A bacteria as before (b). The complex retained by the GF/C filter was eluted with 2% SDS (c). Parallel incubations of the same extract with control sera, treated as for (a), are shown in (d) and (e). All the samples were boiled in sample buffer (10% glycerol, 2% SDS, 0.1 M DTT, 0.05M Tris pH 6.8) and the polypeptides separated by gel electrophoresis on an 8.5% polyacrylamide slab gel. The gel was then impregnated with PPO and radioactivity located by fluorography (15).

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of T antigen appears at 84,000 daltons in these extracts (9), with a minor band at 64,000 relative to the marker polypeptides phosphorylase-a (94,000) and bovine serum albumin (68,000). The other major radiolabeled band present in control as well as test samples is probably actin. Both T bands appear to be collected efficiently by filtration as compared to adsorption to protein A bacteria (8).

DISCUSSION

The assay described here is basically a direct immunoprecipitation, followed by isolation on glass fiber filters of the immune complexes. IgG in the complex is then quantitated by use of iodinated protein A from Staphylococcus aureus. Protein A has a high avidity for IgG from many species (5), is obtainable commercially and readily iodinated. The radiolabeled species is stable for many months at 4°C. This immune complex assay is at least as convenient as any of the others previously reported (10-12) and has several advantages over complement fixation, being as sensitive but more stable and reproducible allowing a more accurate quantitation. It can be used with sera and extracts containing prohibitive amounts of anti-complementary activity or complement, and has the additional advantage that the reactive species of antigen and antibody are isolated on the filter in a convenient form for further characterization. The assay has been used successfully for other antigens (notably X-antigen (13) and mammalian ACT ase) and could in principle be used for any large polyvalent antigen, free or bound to a cell surface. It could also be used for assay of preformed immune complexes, subject to two general limitations. (a) Only those complexes containing immunoglobulin species reactive with protein A will be detected. (b) Only complexes above a certain minimum size and configuration will be retained by the filters. We have not determined the minimum size of complex for retention on glass fiber filters but, using GF/F filters this should be less than 1 micron. Addition of polyethylene glycol (14) during the immunoprecipitation step increases the formation of complexes retained by the filters with low levels of antigen, and should increase the sensitivity and speed of the assay. Other supporting matrices for retention of immune complexes are also being tested now with a view to extending the usable range of immune complex size and facilitating the development of an automated version (4) of the assay.

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