Detection of Simian Virus 40 T-Antigen-related Antigens by a 125I-Protein A-binding Assay and by Immunofluorescence Microscopy on the Surface of SV40-transformed Monolayer Cells

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SUMMARY

Simian virus 40 (SV40)-transformed cells express the SV40-specific tumour transplantation antigen (TSTA) on the cell surface and the SV40-coded tumour antigen in their nuclei. TSTA is defined by SV40-specific transplantation immunity, whereas T-antigen (T-Ag) can be detected serologically by indirect immunofluorescence. Both antigens, however, are derived from the A gene of SV40. We therefore analysed SV40-transformed cells for the presence of serologically detectable T-Ag-related molecules. Such antigens could not be detected on the surface of living SV40-transformed cells in monolayers. However, after a short formaldehyde fixation it was possible to stain the cell surfaces of SV40-transformed cells with sera from rabbits immunized with purified SDS-denatured T-Ag, but not with sera from hamsters bearing SV40-induced tumours. T-Ag-related antigens could be detected with both types of antisera by applying a more sensitive 125I-protein A assay. The T-Ag specificity of the binding of hamster SV40 tumour sera was demonstrated by a 125I-IgG-blocking assay in which preincubation of formaldehyde-fixed SV40-transformed cells with rabbit anti-SDS-T-Ag serum inhibited the binding of hamster SV40 tumour serum by about 70%. The localization of T-Ag-related antigens on the outside of plasma membranes of formaldehyde-fixed cells was shown by an anti-SDS-T-Ag serum-specific binding of fluorescein isothiocyanate-labelled Staphylococcus aureus to the cell surface. Our results are consistent with the hypothesis that SV40 T-Ag-related antigens are involved in the formation of TSTA.

INTRODUCTION

Several simian virus 40 (SV40)-specific antigens have been detected by immunological methods in SV40-transformed cells: large T- and little t-antigen (tumour antigens), U-antigen and TSTA (tumour-specific transplantation antigen) (for review, see Weil, 1978). Large T-antigen (T-Ag) and U-antigen are found by immunofluorescence in nuclei using sera from animals bearing SV40-induced tumours (Pope & Rowe, 1964; Lewis & Rowe, 1971), whereas little t-antigen has been found by immunoprecipitation in the cytoplasm (Prives et al., 1977; Sleigh et al., 1978). In contrast, TSTA is defined by a cell-mediated SV40-specific tumour rejection response in animals immunized with SV40 virus or with SV40-transformed tumour cells (Defendi, 1963; Khera et al., 1963). T-lymphocytes obtained from these animals kill SV40-transformed cells in vitro in a cytotoxicity assay (Gooding, 1977; Trinchieri et al., 1976). In conclusion, to function as an SV40-specific transplantation antigen, TSTA must be localized on the surface of tumour cells.
Although these antigens are defined by different immunological criteria, there is steadily increasing evidence that T-Ag and TSTA are closely related products derived from one single gene locus, the SV40 A gene: (i) nuclei isolated from SV40-transformed cells contain both T-Ag and TSTA activity (Anderson et al., 1977a; Rogers et al., 1977); (ii) certain cells transformed by temperature-sensitive mutants of the A gene of SV40 lose both T-Ag and TSTA when grown at the non-permissive temperature (Anderson et al., 1977b); (iii) T-Ag and TSTA co-purify through different steps of purification (Chang et al., 1977, 1979); (iv) purified T-Ag has been shown to induce an SV40-specific tumour-rejection response in mice (Chang et al., 1979). These observations suggest that either T-Ag and TSTA are located on closely related polypeptides, or that T-Ag itself is involved in the formation of TSTA. Consequently, antisera directed against nuclear T-Ag should detect T-Ag-related antigens on the cell surface. However, until recently (Soule et al., 1980; Henning et al., 1980) attempts to demonstrate T-Ag related molecules serologically on the surface of living SV40-transformed cells have failed (Luborsky et al., 1976; Schmidt-Ullrich et al., 1977; Chang et al., 1979). Recently, it was observed that SV40 T-Ag-related proteins could be detected by immunofluorescence microscopy on the surface of adenovirus–SV40 hybrid virus-infected HeLa cells. However, surface fluorescence was only positive after fixing these cells with formaldehyde (Deppert & Pates, 1979; Deppert & Henning, 1980). Using a short formaldehyde fixation a weak but SV40 T-Ag-specific surface fluorescence could be observed also on SV40-transformed cells with sera from rabbits immunized with purified T-Ag but not with sera from hamsters bearing SV40-induced tumours (Deppert & Henning, 1980; this study). Applying a more sensitive 125I-protein A assay, T-Ag-related molecules have also been detected both with rabbit anti-SDS-T-Ag serum and with hamster SV40 tumour sera. These results suggest that antigens closely related to nuclear SV40 T-Ag occur on the surface of SV40-transformed cells, and might be involved in the formation of TSTA.

**METHODS**

**Cell lines.** Mouse Balb/c fibroblasts (3T3), the corresponding SV40 virus-transformed (SV 3T3) and polyoma virus-transformed cells (Py 3T3) as well as human (SV80) and hamster (H 65/90 B) SV40-transformed cells were grown in minimal essential medium (MEM) or in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 to 10% foetal calf serum.

**Sera.** Two different types of antisera directed against T-Ag were used: rabbit anti-SDS-T-Ag sera and hamster SV40 tumour sera. Antisera directed against purified, SDS-denatured T-Ag were raised in rabbits as described previously (Deppert & Henning, 1980; Deppert & Pates, 1979). Briefly, T-Ag was prepared from 0.5% NP40 extracts (Schwyzer, 1977) of SV80 cells (rabbits no. 20, no. 21) or H 65/90 B cells (rabbit no. 143) by indirect immunoprecipitation and purified by SDS–polyacrylamide gel electrophoresis. T-Ag was eluted from gel slices and injected into rabbits as described elsewhere (Deppert & Henning, 1980; Deppert & Pates, 1979). These rabbit anti-SDS-T-Ag sera had the following titres against nuclear T-Ag: no. 20, titre 1:1600; no. 21, titre 1:3200; no. 143, titre 1:1600.

Hamster SV40 tumour sera were obtained from inbred C'Lak hamsters which were injected subcutaneously with 2 × 10^6 H 65/90 B cells as described by Deppert & Henning (1980). The hamster SV40 tumour sera were pooled according to their immunofluorescence titres against nuclear T-Ag (pool no. 2, titre 1:3200; pool no. 3, titre 1:1000).

The nuclear T-Ag titres of both the rabbit anti-SDS-T-Ag sera and the hamster SV40 tumour sera were determined by immunofluorescence microscopy according to Pope & Rowe (1964) on SV 3T3 cells fixed in absolute ethanol for 30 min at −20 °C using fluorescein isothiocyanate (FITC)-coupled pig anti-hamster IgG or FITC-coupled goat anti-rabbit IgG (Nordic, Byk Mallinckrodt, Dietzenbach-Steinberg, F.R.G.).
Detection of SV40 T-Ag-related surface antigens

Both rabbit anti-SDS-T-Ag sera and hamster SV40 tumour sera were absorbed three times for 12 h on ethanol-fixed confluent 3T3 cells in Petri dishes [1 ml of serum diluted 1:10 in phosphate-buffered saline (PBS) per 9 cm Petri dish].

125I-protein A-binding assay. Protein A binds specifically to the Fc region of most subclasses of mammalian IgG molecules (Kronvall & Frommel, 1970). 125I-labelled protein A therefore can be used as a highly sensitive probe for the detection of antibodies bound to cell surface antigens (for review, see Goding, 1978). A 30 μg amount of protein A (Pharmacia) was labelled with 1 mCi Na125I (NEN) in 0.5 ml PBS by the chloramine-T method according to Hunter & Greenwood (1962). The 125I-labelled protein A was purified by gel chromatography on Sephadex G-25 and had specific activities varying from 1.2 x 107 to 3.1 x 107 ct/min/μg. The 125I-protein A assay was a modification of that described by Brown et al. (1977). Cells (2 x 10^3/well) were seeded in Terasaki microtitre plates. After 6 h the cells were rinsed once with PBS and subsequently fixed with 3.7% formaldehyde in PBS at 0 °C for 4 min and air dried. After washing with PBS 5 μl of antisera or control sera diluted 1:10 in PBS were added to each well for 60 min at 37 °C. The plates were then washed three times for 15 min in PBS, and incubated with approx. 0.02 μg 125I-protein A (corresponding to 2 x 10^6 to 6 x 10^5 ct/min) per well in the presence of 15% bovine serum albumin. After 60 min incubation at 37 °C, excess 125I-protein A was removed by washing the plates extensively for 12 to 16 h at 20 °C until the binding of 125I-protein A to empty wells was reduced to approx. 100 ct/min. The wells containing the labelled cells were cut out of the plates and counted in a gamma counter. Ten out of 60 wells of each Terasaki plate were checked for the percentage of open cells by immunofluorescence staining for nuclear T-Ag, using FITC-coupled goat anti-rabbit IgG or FITC-coupled swine anti-hamster IgG (Nordic).

125I-IgG-blocking assay. To demonstrate the T-Ag specificity of the binding of hamster SV40 tumour sera to the surface of formaldehyde-fixed SV40-transformed cells, a 125I-IgG-blocking assay was performed. Anti-hamster IgG was raised in rabbits immunized four times with 0.5 mg purified hamster IgG in the presence of complete Freund’s adjuvant. A 400 μg amount of purified rabbit anti-hamster IgG was labelled with 1 mCi Na125I (NEN) as described above for the iodination of protein A. The specific activity of the 125I-labelled rabbit anti-hamster IgG was 2.1 x 10^6 ct/min/μg IgG.

SV 3T3 cells (2 x 10^3/well) were seeded in Terasaki microtitre plates and kept at 37 °C overnight. The cells were fixed in 3.7% formaldehyde as described for the 125I-protein A assay and then precoated with 10% bovine serum albumin in PBS for 30 min at 4 °C. After washing the plates five times in PBS, 5 μl blocking antibody (rabbit anti-SDS-T-Ag serum no. 21 or normal rabbit serum diluted 1:10 in PBS) were added to each well and incubated for 60 min at 4 °C. The plates were washed 10 times with PBS and then incubated further with 5 μl/well of the test antibody (hamster SV40 tumour serum no. 3 or normal hamster serum diluted 1:10 in PBS) for 60 min at 4 °C. After another series of washings, each well was incubated with 0.2 μg (4 x 10^5 ct/min) 125I-labelled rabbit anti-hamster IgG in 5 μl PBS for 30 min at 4 °C. Finally, the plates were washed with PBS until the background binding of 125I-IgG to empty wells was reduced to a background of approx. 200 ct/min/well. The wells were then cut out of the plates and counted in a gamma counter.

Immunofluorescence microscopy. Cells were grown on coverslips (12 mm in diam.) in DMEM containing 5 to 10% foetal calf serum depending on the cell line analysed. The cells were fixed on coverslips in 3.7% formaldehyde (1:10 dilution in PBS of 37% stock solution; BDH) for 4 min at 0 °C and air dried as described for the 125I-protein A assay. After air drying the coverslips were washed with PBS and incubated with the first antibody (rabbit anti-SDS-T-Ag sera, hamster SV40 tumour sera, diluted 1:10 in PBS) for 45 min at 37 °C, washed with PBS and incubated with FITC-labelled goat anti-rabbit or swine anti-hamster IgG for a further 45 min at 37 °C. After another series of washes with PBS, the coverslips
were mounted directly with Elvanol on microscopy slides and viewed with a Zeiss microscope (Carl Zeiss, Oberkochen, F.R.G.) equipped with epifluorescent illumination. Pictures were taken with Planapo × 63 oil immersion objectives using automatic exposure timing.

Binding of S. aureus to the cell surface. S. aureus (strain Cowan I) was grown exactly as described by Kessler (1975), inactivated by heating for 30 min at 80 °C and fixed with 1% formaldehyde in PBS at room temperature. Formaldehyde-fixed and heat-inactivated S. aureus (1% in PBS) were coupled to FITC (10 mg/ml) for 30 min at 20 °C and washed several times by centrifugation in order to remove excess FITC. 3T3 or SV 3T3 cells were grown on coverslips, fixed with 3.7% formaldehyde for 4 min at 0 °C and air dried. Alternatively, cells were fixed at 0 °C for 60 min, washed several times with PBS and used without air drying. Both fixation procedures resulted in identical results with respect to the intensity and specificity of the binding of S. aureus to the surfaces of cells precoated with antisera. Formaldehyde-fixed cells were incubated with rabbit anti-SDS-T-Ag sera, or normal rabbit serum respectively for 1 h at 0 to 2 °C. The coverslips were rinsed several times in PBS and incubated according to Ghetie et al. (1974) in a 1% suspension of FITC-coupled S. aureus in PBS for 2 h at 4 °C. Excess S. aureus was removed from the coverslips by washing with PBS. Cells were viewed in a Leitz microscope equipped with epifluorescent illumination using × 16 or × 40 Fluotar objectives.

RESULTS

Immunofluorescence microscopy

The anti-SV40-T-Ag sera described in Methods were used in indirect immunofluorescence microscopy on several living SV40-transformed monolayer cell lines. In no case could a positive cell surface fluorescence be observed. Applying the formaldehyde fixation procedure described previously (Deppert & Pates, 1979) resulted in a highly specific binding of rabbit anti-SDS-T-Ag antibodies to the surface of SV40-transformed cells which could be detected by indirect immunofluorescence microscopy. Cells grown on coverslips were fixed with 3.7% formaldehyde for 4 min at 0 °C and analysed for surface fluorescence using both rabbit anti-SDS-T-Ag sera or hamster SV40 tumour sera after absorption on ethanol-fixed 3T3 cells as described in Methods. All three rabbit anti-SDS-T-Ag sera listed in Table 1 were positive in immunofluorescence analysis on SV80 and on SV 3T3 but not on 3T3 nor on Py 3T3 cells. As an example, Fig. 1(a, b) shows formaldehyde-fixed SV 3T3 cells stained with rabbit anti-SDS-T-Ag serum (no. 143) and FITC-coupled goat anti-rabbit IgG. Normal rabbit serum did not stain the surface of these cells. Neither 3T3 (Fig. 1c, d) nor polyoma virus-transformed 3T3 (Py 3T3) cells (data not shown) showed positive surface fluorescence using the same rabbit anti-SDS-T-Ag sera.

In general, the SV40-specific cell surface fluorescence shown in Fig. 1 was seen on 60 to 90% of SV40-transformed cells with the rabbit anti-SDS-T-Ag sera. However, the titres of the anti-SDS-T-Ag sera were weak (1:20). Additionally, none of the available hamster SV40 tumour sera gave positive surface fluorescence. To check whether this might be due to a threshold problem, we applied the 125I-protein A-binding assay as a method with possibly higher sensitivity.

125I-protein A assay

To compare the sensitivity of the 125I-protein A assay with that of immunofluorescence microscopy, two rabbit anti-SDS-T-Ag sera (no. 20, no. 143) and two hamster SV40 tumour sera were titrated by both methods for reaction with surface T-antigenic binding sites. Both hamster SV40 tumour sera were negative in surface fluorescence, while with the 125I-protein A assay these sera were positive up to a dilution of 1:40. The rabbit anti-SDS-T-Ag sera no. 20
Detection of SV40 T-Ag-related surface antigens

Fig. 1. Cell surface fluorescence analysis of SV 3T3 and 3T3 cells using rabbit anti-SDS-T-Ag serum. SV 3T3 and 3T3 cells grown on coverslips were fixed with formaldehyde and stained for indirect immunofluorescence microscopy using rabbit anti-SDS-T-Ag serum no. 143, diluted 1:10 with PBS. (a) SV 3T3 cells, fluorescence picture; (b) corresponding phase picture; (c) 3T3 cells, fluorescence picture; (d) corresponding phase picture. Similar fluorescence pictures were obtained with the other rabbit anti-SDS-T-Ag sera (no. 20, no. 21). Normal rabbit serum was fluorescence-negative on both SV 3T3 and 3T3 cells. Exposure times: (a) 30 s; (c) 2.5 min. All bar markers represent 25 μm.

and no. 143 were positive for surface fluorescence only up to a 1:20 dilution, while in the $^{125}$I-protein A assay, they were routinely positive up to a 1:80 dilution. These results indicate that, under our experimental conditions, the $^{125}$I-protein A assay provides higher sensitivity than immunofluorescence microscopy. As an example, Fig. 2 shows the titration curves of rabbit anti-SDS-T-Ag serum no. 20 and preimmune serum no. 20 on formaldehyde-fixed SV 3T3 cells in the $^{125}$I-protein A assay (titre 1:80).

Table 1 summarizes representative results obtained in the $^{125}$I-protein A assay using five different antisera on four cell lines. Three (no. 20, no. 21, no. 143) out of four individual rabbit anti-SDS-T-Ag sera reproducibly showed significantly positive reactions on SV40-transformed cells when compared with 3T3 or Py 3T3 cells. To point out the specificity of the binding of anti-T-Ag antibodies to SV40-transformed cells the ‘T-Ag-specific binding’ (see
Fig. 2. Titration of rabbit anti-SDS-T-Ag serum no. 20 and normal rabbit serum in the $^{125}$I-protein A assay on formaldehyde-fixed SV 3T3 cells. The results are given as the numbers of $^{125}$I-protein A molecules ($\times 10^{-6}$) bound to formaldehyde-fixed SV 3T3 cells precoated with rabbit anti-SDS-T-Ag serum no. 143 or normal rabbit serum/well of Terasaki microtitre plates. The wells were seeded with $2 \times 10^3$ cells. Each point represents the average value obtained from 10 wells including the standard deviation.

Table 1. $^{125}$I-protein A assay on formaldehyde-fixed cells*

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>SV80</th>
<th>SV 3T3</th>
<th>3T3</th>
<th>Py 3T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-SDS-T-Ag no. 20</td>
<td>674 ± 64</td>
<td>473 ± 84</td>
<td>275 ± 33</td>
<td>537 ± 59</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>353 ± 75</td>
<td>114 ± 30</td>
<td>165 ± 33</td>
<td>632 ± 104</td>
</tr>
<tr>
<td>T-Ag specific binding†</td>
<td>321 ± 98</td>
<td>359 ± 89</td>
<td>110 ± 46</td>
<td>-95 ± 119</td>
</tr>
<tr>
<td>Rabbit anti-SDS-T-Ag no. 21</td>
<td>512 ± 96</td>
<td>554 ± 48</td>
<td>282 ± 27</td>
<td>538 ± 85</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>204 ± 70</td>
<td>120 ± 15</td>
<td>165 ± 22</td>
<td>469 ± 28</td>
</tr>
<tr>
<td>T-Ag specific binding</td>
<td>308 ± 118</td>
<td>434 ± 50</td>
<td>117 ± 34</td>
<td>69 ± 89</td>
</tr>
<tr>
<td>Rabbit anti-SDS-T-Ag no. 143</td>
<td>842 ± 158</td>
<td>681 ± 56</td>
<td>221 ± 34</td>
<td>682 ± 52</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>490 ± 64</td>
<td>202 ± 19</td>
<td>135 ± 17</td>
<td>641 ± 64</td>
</tr>
<tr>
<td>T-Ag specific binding</td>
<td>352 ± 170</td>
<td>479 ± 59</td>
<td>66 ± 38</td>
<td>41 ± 82</td>
</tr>
<tr>
<td>Hamster serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hamster tumour serum, pool no. 2</td>
<td>444 ± 40</td>
<td>395 ± 27</td>
<td>166 ± 42</td>
<td>166 ± 25</td>
</tr>
<tr>
<td>Normal hamster serum</td>
<td>186 ± 21</td>
<td>163 ± 14</td>
<td>277 ± 36</td>
<td>190 ± 26</td>
</tr>
<tr>
<td>T-Ag specific binding</td>
<td>258 ± 45</td>
<td>232 ± 30</td>
<td>-111 ± 55</td>
<td>-24 ± 36</td>
</tr>
<tr>
<td>Hamster tumour serum, pool no. 3</td>
<td>241 ± 29</td>
<td>231 ± 15</td>
<td>177 ± 25</td>
<td>180 ± 24</td>
</tr>
<tr>
<td>Normal hamster serum</td>
<td>110 ± 13</td>
<td>96 ± 27</td>
<td>134 ± 34</td>
<td>132 ± 14</td>
</tr>
<tr>
<td>T-Ag specific binding</td>
<td>131 ± 31</td>
<td>135 ± 30</td>
<td>43 ± 42</td>
<td>48 ± 31</td>
</tr>
</tbody>
</table>

* The results represent the mean ± standard deviation (n = 6) of the number of $^{125}$I-protein A molecules ($\times 10^{-6}$) bound/one microtitre plate well. Each well contained $2 \times 10^3$ cells. The data were calculated from the specific radioactivity of $^{125}$I-protein A used for the particular experiment and from the $^{125}$I ct/min/well. All sera were used at a dilution of 1:10.

† T-Ag-specific binding was estimated by the formula:

$$IS = NS \pm \sqrt{s.d. IS^2 + s.d. NS^2}$$

where IS is the number of protein A molecules bound to cells incubated with immune serum, and NS the number of protein A molecules bound to cells incubated with normal serum; s.d., standard deviation.

Table 1) was calculated by subtraction of the data obtained with normal sera from the data obtained with immune sera. The calculation of the significance of the 'T-Ag-specific-binding' data in a Student's $t$-test in all cases clearly showed significant differences ($2P < 0.005$ to $0.001$) between SV40-transformed cells and the two other cell lines (Py 3T3, 3T3). No significant differences in the T-Ag-specific binding could be observed between SV80 and SV 3T3 ($P < 0.05$ to $0.25$) as well as between 3T3 and Py 3T3 ($2P < 0.05$ to $0.5$).
Detection of SV40 T-Ag-related surface antigens

Table 2. $^{125}$I-IgG-blocking assay on formaldehyde-fixed SV 3T3 cells*

<table>
<thead>
<tr>
<th>Blocking antibody</th>
<th>Test antibody</th>
<th>Binding of $^{125}$I-IgG to test antibody (ct/min)</th>
<th>Specific binding of test antibody†</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Hamster tumour serum, no. 3</td>
<td>509 ± 44</td>
<td>224 ± 50</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>Normal hamster serum</td>
<td>285 ± 25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>Hamster tumour serum, no. 3</td>
<td>495 ± 52</td>
<td>200 ± 57</td>
<td>70</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>Normal hamster serum</td>
<td>295 ± 34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit anti-SDS-T-Ag no. 21</td>
<td>Hamster tumour serum, no. 3</td>
<td>400 ± 81</td>
<td>60 ± 82</td>
<td></td>
</tr>
<tr>
<td>Rabbit anti-SDS-T-Ag no. 21</td>
<td>Normal hamster serum</td>
<td>340 ± 17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* SV 3T3 cells were serially incubated with 1:10 dilutions of the blocking antibody (rabbit anti-SDS-T-Ag serum no. 21 or normal rabbit serum), 1:10 dilutions of the test antibody (hamster SV40 tumour serum no. 3 or normal hamster serum) and finally with $^{125}$I-labelled anti-hamster IgG as described in Methods. The results represent the mean ± standard deviation (n = 6) of $^{125}$I ct/min counted/one microtitre plate well.

† Specific binding of test antibody was estimated by the formula:

$$IS - NS \pm \sqrt{S.D. IS^2 + S.D. NS^2}$$

where IS is the ct/min obtained with hamster SV40 tumour serum as test antibody, and NS the ct/min obtained with normal hamster serum as test antibody; S.D., standard deviation.

The majority of T-Ag found in SV40-transformed cells resides in the nuclei. Therefore, it was extremely important to examine whether anti-T-Ag antibodies were able to bind to nuclear T-Ag in formaldehyde-fixed cells. This was checked routinely with the $^{125}$I-protein A assay by counting the percentage of open cells which were positive for nuclear T-Ag staining by immunofluorescence in 10% of the wells: when the temperature during formaldehyde fixation was kept exactly at 0 °C, no more than 0.05% (on average 1 cell/well) of the formaldehyde-fixed cells were positive for nuclear T-Ag staining. However, after fixing the cells at room temperature, approx. 50% of the cells became positive for nuclear T-Ag in immunofluorescence microscopy. Under these experimental conditions, additional binding of antibodies to nuclear T-Ag-positive cells increased the results of the $^{125}$I-protein A assay only by a factor of approx. 10 (data not shown). From these data one can calculate that the binding of T-Ag antibodies to 0.05% nuclear T-Ag-positive cells could in no case have caused the results listed in Table 1. We therefore conclude that the formaldehyde fixation procedure used in this study did not induce antibody permeability in the cells. Similar results concerning formaldehyde fixation were reported by Mautner & Hynes (1977) and by Deppert & Pates (1979).

$^{125}$I-IgG-blocking assay

The results obtained in the $^{125}$I-protein A assay indicate that T-Ag-related binding sites could be detected on the surface of formaldehyde-fixed SV40-transformed cells with rabbit anti-T-Ag sera as well as with hamster SV40 tumour sera. Since hamster SV40 tumour sera detect not only SV40 T-Ag but also host cell-coded proteins (Häyri & Defendi, 1970; Linzer & Levine, 1979) the T-Ag specificity of the binding of the tumour sera in the $^{125}$I-protein A assay had to be confirmed. We therefore determined, in a $^{125}$I-IgG-blocking assay, whether the rabbit anti-T-Ag sera, i.e. sera with a proven specificity for T-Ag-related binding sites (Deppert & Pates, 1979; Deppert & Henning, 1980), were able to block the binding of hamster SV40 tumour sera to the cell surfaces of SV40-transformed cells. Formaldehyde-fixed SV 3T3 cells, precoated with rabbit anti-SDS-T-Ag serum or normal rabbit serum, were incubated with hamster SV40 tumour serum or normal hamster serum. The binding of the hamster sera was measured using $^{125}$I-labelled rabbit anti-hamster IgG (see Methods). As shown in Table 2, preincubation of formaldehyde-fixed SV 3T3 cells with rabbit anti-SDS-T-Ag serum no. 21 inhibited the binding of hamster SV40 tumour serum no. 3 by 70%.
Fig. 3. Binding of FITC-labelled *S. aureus* on the surface of SV 3T3 cells precoated with either rabbit anti-SDS-T-Ag serum or rabbit normal serum. SV 3T3 cells were fixed with formaldehyde, precoated with either rabbit anti-SDS-T-Ag serum no. 20 (a, b) or normal rabbit serum (c, d), and then stained with FITC-labelled *S. aureus* as described in Methods. (a) SV 3T3 cells, precoated with rabbit anti-SDS-T-Ag serum, fluorescence picture; (b) corresponding phase picture; (c) SV 3T3 cells, precoated with normal rabbit serum, fluorescence picture; (d) corresponding phase picture. Exposure times: 15 s for (a) and (c). All bar markers represent 50 μm.

**Binding of *S. aureus* to the cell surface of formaldehyde-fixed cells**

Although the immunofluorescence studies had demonstrated the location of binding sites for antibodies directed against T-Ag on the surface of formaldehyde-fixed cells, this method cannot unambiguously demonstrate the location of these binding sites on the outer surface of the plasma membrane. To show more directly the location of T-Ag-related surface antigens on the outer surface of the plasma membrane of formaldehyde-fixed cells, we analysed the binding of FITC-labelled *S. aureus* on formaldehyde-fixed SV 3T3, 3T3 and Py 3T3 cells which were precoated with rabbit anti-SDS-T-Ag serum no. 143 or with normal rabbit serum. *S. aureus* carries protein A on the outside of its cell wall and, therefore, should be able to demonstrate the presence of antigen–antibody complexes on the outer surface of the plasma membrane of formaldehyde-fixed cells. As demonstrated in Fig. 3, FITC-coupled *S. aureus*
Detection of SV40 T-Ag-related surface antigens

bound only to the surface of formaldehyde-fixed SV 3T3 cells precoated with rabbit anti-SDS-T-Ag serum (Fig. 3a, b), but not to SV 3T3 cells precoated with normal rabbit serum (Fig. 3c, d). FITC—S. aureus binding was not observed on formaldehyde-fixed 3T3 or on Py 3T3 cells precoated with rabbit anti-SDS-T-Ag sera or with normal sera (data not shown). These data suggest that SV40 T-Ag-related antigens, as detected by immunofluorescence microscopy and by the 125I-protein A assay, are in fact located at least in part on the outer surface of formaldehyde-fixed SV40-transformed cells.

Discussion

Several experimental observations indicate the presence of SV40 T-Ag, or very closely related antigens, on the surface of SV40-transformed cells. Very recently, T-Ag-like antigens were detected by immunofluorescence on the surface of living SV40-transformed monolayer cells put into suspension either by treatment with EDTA (Soule et al., 1980) or mechanically (Henning et al., 1980). However, in our hands all the anti-T-Ag sera used so far were negative in immunofluorescence microscopy on living SV40-transformed monolayer cells in situ. Similar observations were reported for HeLa cells infected with adenovirus—SV40 hybrid virus Ad2+ND1 and Ad2+ND2 (Deppert & Pates, 1979), although, for these cells it had been shown that the SV40-specific hybrid virus proteins accumulated in the plasma membrane during the infection (Deppert & Walter, 1976). For a successful serological detection of these proteins on the cell surface a short formaldehyde fixation had to be applied in order to change the surface architecture. Since after this treatment the SV40-specific proteins could be visualized by immunofluorescence we applied this formaldehyde fixation procedure to SV40-transformed monolayer cells and found a weak but SV40 T-Ag-specific surface fluorescence on these cells in situ using rabbit anti-SDS-T-Ag sera (Deppert & Henning, 1980; Deppert et al., 1980). In contrast, hamster SV40 tumour sera did not stain the cell surfaces of formaldehyde-fixed cells, although these tumour sera had similar high titres against nuclear T-Ag. These observations might be due to the exposure by formaldehyde of antigenic sites of surface T-Ag not exposed on nuclear T-Ag, and which therefore cannot be detected with hamster SV40 tumour sera. Alternatively, surface T-Ag exhibits, at least in part, the same antigenic binding sites as nuclear T-Ag and the negative results obtained in immunofluorescence with hamster SV40 tumour sera might then be due to a threshold problem. To substantiate and extend our results obtained by immunofluorescence microscopy, we set up a 125I-protein A-binding assay as a significantly more sensitive tool for the detection of T-Ag-related antigens on the surface of SV40-transformed cells.

As can be seen in Table 1, the ‘T-Ag-specific-binding’ of these rabbit anti-SDS-T-Ag sera were positive on two SV40-transformed cell lines of different species when compared with normal 3T3 cells or with polyoma-transformed 3T3 cells. The statistical calculations in all cases showed significant differences between SV40-transformed (SV80, SV 3T3) and non-SV40-transformed (Py 3T3) or untransformed cells (3T3). The titres of the rabbit anti-SDS-T-Ag sera (1:80) were significantly higher in the radioimmunoassay when compared with the immunofluorescence experiments (1:20). In contrast to the negative results obtained by immunofluorescence microscopy, SV40-specific binding of hamster SV40 tumour sera could be detected by applying the more sensitive 125I-protein A assay on the surfaces of formaldehyde-fixed cells, although the titres of the hamster tumour sera (1:20) were lower than the titres of the rabbit anti-SDS-T-Ag sera (1:80). The positive results obtained with hamster SV40 tumour sera in the radioimmunoassay suggest that the failure of these sera to stain cell surfaces by immunofluorescence microscopy more likely reflects a lower sensitivity of the immunofluorescence assay than inability of the tumour sera to recognize T-antigenic binding sites of surface T.
The establishment of a relationship of the detected cell surface antigens with nuclear SV40 T-Ag depends on the specificity of the antisera used. Consequently, the specificity of the antisera used is most important for the interpretation of the results in the present study. The rabbit anti-SDS-T-Ag sera have been raised against purified, SDS-denatured T-Ag isolated from human (SV80) or from hamster (H 65/90 B) SV40-transformed cells.

In addition, the rabbit anti-SDS-T-Ag sera, as well as the hamster SV40 tumour sera, reproducibly precipitated large T-Ag from 3H-leucine-labelled extracts from SV40-transformed cells (SV80, SV 3T3) and, dependent on the amounts of antisera and S. aureus used for immunoprecipitation, middle T (non-viral T-antigen, NVT) and little t could also be observed on SDS-polyacrylamide gels. Except for NVT, no host cell protein was precipitated in detectable amounts either from SV40-transformed or from 3T3 cells (Deppert & Henning, 1980). According to recent reports, the co-precipitation of large T-Ag and NVT by anti-T-Ag sera is caused by the formation of a complex between T-Ag and NVT rather than by an antigenic relationship (Lane & Crawford, 1979). Therefore, one can conclude that anti-T-Ag sera react only with T-Ag, little t-Ag or with closely related antigens. Finally, most important for the interpretation of the data obtained with hamster SV40 tumour sera, the binding of these sera to formaldehyde-fixed cells could be inhibited up to 70% by precoating these cells with rabbit anti-T-Ag sera. Altogether, these data strongly suggest that the antigens detected on the surface of formaldehyde-fixed cells are closely related to nuclear T-Ag.

Although the formaldehyde fixation used had the distinct advantage of exposing T-Ag on the cell surface, several precautions had to be taken into account in order to interpret our data. First, to demonstrate the surface specificity of the radioimmunoassay the serological reaction had to be visualized by immunofluorescence microscopy. Second, under our experimental conditions (0 °C, 4 min) the nuclei were not stained, indicating that the cell membrane did not become permeable to antibodies. Therefore, one can presume that in the 125I-protein A assay and immunofluorescence microscopy we probably detected only a binding reaction of the anti-T-Ag sera on or at least at the level of the plasma membrane. However, it is not possible to decide by these methods whether these antigens are located outside, within or even inside the plasma membrane. Since, due to their size, S. aureus bacteria cannot penetrate the plasma membrane, the binding of FITC-labelled S. aureus to formaldehyde-fixed cells, precoated with rabbit anti-T-Ag serum, provided direct evidence that T-Ag-related molecules must, at least in part, be located on the outside of the plasma membrane.

In conclusion, the effects of formaldehyde can be interpreted in at least two ways: formaldehyde treatment either fixes SV40 T-Ag-related antigens which are normally only loosely associated with the cell surface, or it unmasks T-Ag binding sites on the cell surface by changing the cell surface architecture. The latter interpretation would best explain our data; T-Ag-related molecules are present on the surface of living monolayer cells but they are only partially accessible to antibodies. Formaldehyde could unmask T-Ag-related molecules so that more antigenic binding sites become accessible to T-Ag antibodies. This hypothesis has recently been substantiated and extended by experiments on living SV40-transformed monolayer cells in situ. Using the highly sensitive 125I-protein A assay, low levels of T-Ag-related binding sites were detected with rabbit anti-SDS-T-Ag sera as well as with hamster SV40 tumour sera. As in the present study, both types of antisera did not stain the surface in the less sensitive immunofluorescence assay (Henning et al., 1980). In conclusion, T-Ag-related binding sites, closely related to nuclear T-Ag, exist on the surface of SV40-transformed cells. These data are consistent with the hypothesis that T-Ag or closely related antigens are involved in the formation of TSTA.

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