The Immune Response Against the ASV-coded src-Gene Product in Syngeneic Mice

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SUMMARY

The antigenicity of the avian sarcoma virus (ASV)-coded src-gene product pp60src, which is responsible for fibroblast transformation after ASV infection, has been investigated in STU mouse fibrosarcoma cell lines and the corresponding immune response in syngeneic mice has been determined. The development of effective anti-pp60src antibody titres depends on the mode and site of injection of tumour cells and parallels tumour growth. It was found that mouse immunoglobulin heavy chains are unable to serve as substrate for the protein kinase activity of pp60src. Therefore, an indirect protein kinase absorption (PKA) test was initiated to demonstrate recognition of the protein kinase activity associated with the src-gene product. The availability of syngeneic mice and the corresponding ASV-transformed tumour cells should facilitate studies designed to elucidate the possible relationship between the cytoplasmic pp60src and ASV-induced tumour-specific surface antigens (TSSA), for example, by allowing the production of stable mouse hybridomas synthesizing antibodies specific for pp60src and TSSA.

One of the best studied agents which transform normal cells are the avian sarcoma viruses (ASV). The gene responsible for neoplastic transformation of chicken embryo fibroblasts (CEF) is denoted src (for review, see Vogt, 1977). The product of the src-gene was identified using antisera from rabbits bearing tumours induced by the ASV strain, Schmidt-Ruppin D (SR-D). This molecule is a phosphoprotein with a mol. wt. of 60,000 (pp60src) which exhibits an IgG-heavy chain phosphorylating activity in the immune precipitate (Brugge & Erikson, 1977; Brugge et al. 1978a; Collett & Erikson, 1978; Levinson et al. 1978; Purchio et al. 1978; Sefton et al. 1978). Conditional mutations in the src-gene affect both enzyme activity and thermal stability of pp60src (Collett & Erikson, 1978; Levinson et al. 1978; Rübsamen et al. 1979), providing evidence that the enzymic activity is a true function of pp60src and not a contamination in the precipitate.

In the present study we have characterized the immune reaction to pp60src in syngeneic mice bearing SR-D-derived tumours. This species is of special interest for us as we have initiated the establishment of mouse-mouse hybridomas for the production of monoclonal antibodies against pp60src using the method described by Köhler & Milstein (1975). Anti-pp60src antisera and corresponding monoclonal antibodies will be used for elucidation of possible relationships between pp60src and the tumour-specific cell surface antigen (TSSA) known to be associated with ASV-induced cell transformation (for review, see Kurth & Bauer, 1975; Kurth et al. 1979).

Chicken embryo fibroblasts (CEF) were prepared from 11 day-old embryos (Spafas Inc., Roanoke, Ill., U.S.A.) and infected with the ASV strain SR-D (originally obtained from R. R. Friis, Giessen University, West Germany) or a transformation-defective (td) SR-D deletion mutant (donated by T. Graf, Max-Planck-Institut für Virusforschung, Tübingen, West Germany). CEF infected with td SR-D virus were subcultured at least three to four
times. Successful infection was repeatedly monitored by interference tests (Vogt & Ishizaki, 1966).

The ASV-transformed STU mouse cell lines D17 (a gift from G. Pauli, Giessen University) and D56 (established by L. Netuschil, Friedrich-Miescher-Laboratorium, Max-Planck-Institute) were employed as potential sources of pp60<sup>src</sup> from mouse cells. D17 fibrosarcoma cells are derived from an <em>in vitro</em> infection of STU mouse embryo fibroblasts with SR-D virus. D56 cells stem from an explanted tumour induced by SR-D virus in a newborn mouse.

Rabbit antisera against pp60<sup>src</sup> were prepared as described by Brugge & Erikson (1977). In mice (mouse strain STU; Schäfer, 1979), tumours were induced either by subcutaneous injection of purified SR-D into newborns (as in rabbits) or by injection of living syngeneic SR-D-transformed mouse cells (D17 or D56) into 6 month-old mice. Sera were collected at the times indicated in Fig. 1 and Table 1. Antisera against ASV structural proteins were kindly prepared by J. Löwer (Friedrich-Miescher-Laboratorium, Max-Planck-Institute).

To prepare radioactive lysates for immunoprecipitation, cells were seeded in Costar plates (Costar, Cambridge, Mass., U.S.A.) at a concentration of 5 × 10<sup>5</sup> cells per 2 cm<sup>2</sup> well. One day later, the subconfluent cultures were starved for 30 min in methionine-lacking medium and subsequently labelled for 2 h with 150 μCi 35S-methionine (300 to 800 Ci/mmol; Radiochemical Centre, Amersham, U.K.) per well in Eagle’s medium lacking methionine and supplemented with 10% dialysed foetal calf serum. Labelled cells were washed twice with normal growth medium and could be stored for lysate preparation at −70 °C for up to 4 weeks. Non-labelled cells were similarly stored without loss of ASV-specific protein kinase activity.

For pp60<sup>src</sup> precipitation, cells were lysed by addition of 0.5 ml/well of lysis buffer A [10 mM-tris-HCl, pH 7.2, 1% Nonidet P40 (NP40), 0.1% desoxycholate (DOC), 0.1 mM-NaCl, 1 mM-phenylmethylsulphonyl fluoride (PMSF), 0.1% Trasylol, 0.5 mg/ml myoglobin]. Lysates were clarified by centrifugation at 12,000 g for 10 min.

To remove precursors to ASV internal core proteins, lysates were treated for 30 min at 4 °C with anti-ASV antiserum (5 μl per 100 μl lysate). The rabbit IgG was then precipitated with protein A-containing <em>Staphylococcus aureus</em> (Kessler, 1975). The absorbed lysate was clarified further by centrifugation at 100,000 g for 1 h. For immunoprecipitation of 35S-methionine-labelled pp60<sup>src</sup>, approx. 3 × 10<sup>6</sup> TCA-precipitable counts of the pre-absorbed supernatant were incubated with 5 μl of normal mouse serum or tumour-bearing mouse (TBM) serum. After 1 h at 4 °C, immune complexes were collected by addition of <em>S. aureus</em>. The resulting pellet was washed twice with buffer A and twice with buffer A containing 1 M-NaCl without DOC. The final pellet was suspended in sample buffer according to Laemmli (1970). After boiling for 3 min, the bacteria were removed by centrifugation and eluted proteins were separated on an SDS-polyacrylamide slab gel using a gradient of 7.5 to 15% acrylamide.

A proportion of sera from individual tumour bearing mice precipitated a 60K protein specific for ASV-induced cell transformation from extracts of 35S-methionine-labelled, SR-D-transformed CEF (Fig. 1a) as well as of SR-D-transformed mouse cells, D17 or D56 (data not shown). Such immune response could be obtained from mice either inoculated as newborns with intact SR-D virus or injected as adults with live, syngeneic D17 (Fig. 1a) or D56 tumour cells (data not shown).

As mentioned above, the protein kinase enzyme activity of the src-gene product phosphorylates <em>in vitro</em> the heavy chain of anti-pp60<sup>src</sup> rabbit antibodies. Surprisingly, we could not detect a corresponding enzyme activity whenever mouse sera were employed to precipitate pp60<sup>src</sup> (data not shown).
**Short communications**

Fig. 1. Immunoprecipitation of the src-gene product from SR-D-transformed cells. (a) Autoradiogram of immunoprecipitated proteins from lysates of 35S-methionine-labelled td SR-D-infected (tracks 1 and 3) and SR-D-infected (tracks 2, 4 and 5) CEF. Precipitation was carried out using TBM antiserum (tracks 1 and 2) taken from a 12 week-old mouse which had been injected when newborn with SR-D virus, TBM-antiserum pool (tracks 3 and 4) containing sera of 10 mice which were bled 7 weeks after the initial injection of 6 × 10^6 D17 cells into their tails, and normal mouse serum (track 5). Mol. wt. markers were: bovine serum albumin (68000), ovalbumin (43000), carbonic anhydrase (29000), myoglobin (17000) and cytochrome c (12000). Arrowheads indicate pp60^src. (b) Protein kinase absorption test (PKA test) with sera of tumour-bearing mice. All sera were reacted with the same amount of D17 cell lysate (corresponding to 10^6 cells). Lysate samples were pre-treated with different mouse sera before exposure to anti-pp60^src rabbit antiserum and subsequent protein kinase reaction. Pre-treatment was done with normal mouse serum (track 1), TBM antiserum pool (track 2) and sera of 14 individual mice (tracks 3 to 16) with tumours of different sizes, bleedings taken 7 weeks after the initial injection of 6 × 10^6 D17 cells into the tails. The arrowhead indicates the position of phosphorylated rabbit IgG heavy chains.

As *S. aureus* bacteria bind only certain subclasses of mouse IgG (Kronvall *et al.* 1970) it was possible that these mouse immunoglobulins being able to serve as substrate for the pp60^src-associated protein kinase activity might have remained in the supernatant. However, using a double immunoprecipitation procedure with a rabbit antiserum specific for all mouse IgG subclasses, we were again unable to demonstrate the protein kinase activity of the pp60^src recognized by the TBM sera. On the other hand, phosphorylation of anti-pp60^src-specific rabbit antibodies could easily be achieved with immune complexes either bound to *S. aureus* (see Fig. 1b) or precipitated by goat anti-rabbit IgG antiserum (not shown).

To answer the question whether antibodies in TBM sera were either not phosphorylated by pp60^src or whether they did not precipitate the pp60^src-associated protein kinase activity, a protein kinase absorption test (PKA test) was initiated. First, the lysates of SR-D-transformed chicken or mouse cells were incubated with the different mouse sera under study. Second, mouse IgG, to which pp60^src may have been bound, was removed using *S. aureus*. The remaining supernatant was subsequently tested for residual protein kinase activity using a known anti-pp60^src rabbit antiserum in the direct kinase assay.

In a typical experiment, 10^7 cells were lysed with 1 ml of buffer B (175 mm-sodium phosphate, pH 7.2, 1%, NP40, 0.1% DOC, 40 mm-NaF, 1 mm-PMSF, 1% Trasylol). After clarification of the cell lysate (10000 g for 1 h), 100 μl lysate and 10 μl of normal or TBM serum were incubated for 1 h at 4 °C. After adsorption of the IgG to *S. aureus* and sedimentation of the complexes, the remaining supernatant was reacted with 5 μl of a previously characterized anti-pp60^src serum from a tumour-bearing rabbit (TBR). Immune complexes were bound to *S. aureus* and washed as described for the precipitation of 35S-methionine-labelled pp60^src using buffer B instead of A. After a final wash with kinase reaction buffer [20 mm-(N-morpholino)ethanesulphonic acid (MES), pH 6.5, 30 mm-MgCl₂] the pellet
was resuspended in 20 µl of this buffer. The enzyme reaction was started by addition of 10 µl (10 µCi) ³²P-γ-ATP (> 2000 Ci/mmol; Radiochemical Centre, Amersham). After a 1 min incubation period at room temperature the reaction was stopped by adding 30 µl of double-concentrated electrophoresis sample buffer (Laemmli, 1970) followed by boiling and analysis of phosphorylated proteins on a SDS-containing 10% acrylamide slab gel.

Fig. 1(b) shows that the 60K precipitating mouse sera did absorb all or most of the protein kinase activity (e.g. in tracks 3 to 6), depending on their individual titres. For the apparent inability of pp60⁻⁰ to phosphorylate mouse IgG, two explanations were possible: (i) the antibodies were blocking the active site of the enzyme or (ii) the mouse IgG heavy chain was not serving as a substrate for the phosphorylation by pp60⁻⁰. We think that the latter explanation is more likely. First, among the many mouse sera tested, there should have been at least some with intermediate to low titres lacking antibodies to the active site of the enzyme, thus allowing phosphorylation of the TBM-IgG. Second, it should be kept in mind that the pp60⁻⁰-dependent, heavy-chain phosphorylation, although practically very useful, is biologically a highly artificial reaction. We therefore conclude that under the experimental conditions described here, mouse immunoglobulins cannot serve as acceptor for the pp60⁻⁰-mediated phosphorylation. The development of the PKA test presented in this communication circumvents this impractical property of mouse IgG.

To find an effective and reproducible immunization procedure, we looked at the differences in the immune response of individual mice. The question was whether there is any correlation between the mode of tumour induction, time of tumour development post-immunization, tumour size and antibody titres against pp60⁻⁰.

Groups of ten mice were each injected in the neck with 6 × 10⁵ or 6 × 10⁴ SR-D-transformed syngeneic mouse cells (D17 or D56). All animals developed rapidly growing fibrosarcomas to which they succumbed after 2 to 3 weeks. However, only two of the animals developed an appreciable anti-pp60⁻⁰ titre (data not shown).

Other groups of mice were injected with 6 × 10⁵ or 6 × 10⁴ D17 or D56 cells into the tail. After extended latency periods of 3 to 6 weeks, tumours again developed in all cases. In this case, a positive correlation between tumour size and antibody titre could be observed. Most mice with large sarcomas (about 2 cm in diam.) developed easily demonstrable anti-pp60⁻⁰ titres (Fig. 1 b, e.g. tracks 3 to 9, except 7). However, there were also notable exceptions, as shown, e.g., in tracks 7 and 10. The sera with lower titres (tracks 11 to 16, except 13) are, on average, from mice with smaller tumours (< 1 cm in diam.).

The time course of tumour development and the corresponding anti-pp60⁻⁰ immune response was also correlated. Table 1 shows that 2 weeks after the booster of group I mice with 6 × 10⁵ cells, when the tumours became easily visible, antibody production against pp60⁻⁰ started and continuous growth of the tumours was paralleled by an increase in antibody titres. Three weeks later, when the tumours reached diam. of over 2 cm and the animals began to die, we often observed a decrease in the antibody titres. The second group showed an analogous picture (Table 1). Since the mice in this group received a booster injection of less D17 tumour cells (6 × 10⁴), their tumours developed 2 to 3 weeks later. Consequently, their anti-pp60⁻⁰ immune response also developed 2 to 3 weeks later compared to group I mice. These data, taken together, suggest that the slow development of large sarcomas favours an effective anti-pp60⁻⁰ immune response.

We have recently obtained evidence that a proportion of the pp60⁻⁰-specific mouse sera react with the ASV-induced tumour-specific cell-surface antigen (TSSA) in microcytotoxicity and immunofluorescence assays (T. Tanaka, J. Stegmann and R. Kurth, unpublished data). Although initially it was found that pp60⁻⁰ is predominantly located in the cytoplasm (Brugge et al. 1978 b; Rohrschneider, 1979), there is recent evidence for location of this
Table 1. Time scale of the anti-pp60<sup>re</sup> immune response in mice injected with D17 cells into the tails*

<table>
<thead>
<tr>
<th>No. of individual mice</th>
<th>Weeks after first injection of D17 tumour cells†</th>
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<tr>
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<td>Group I: injection and booster with 6 × 10&lt;sup&gt;5&lt;/sup&gt; cells‡</td>
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* The antibody titres of individual mouse bleedings against pp60<sup>re</sup> were determined by the PKA test using D17 cell lysates. The enzyme activity remaining in the pre-treated supernatant was determined by immunoprecipitation with anti-pp60<sup>re</sup> rabbit antiserum.
† A booster injection was given in the third week after the first injection.
‡ - -, No removal of protein kinase activity from the transformed cell lysate: +, just visible removal; ++, significant removal; ++++, complete removal (compare with Fig. 1 b).
§ NT, Not tested.
|| This mouse died.

protein at or near the plasma membrane (Willingham et al. 1979). Therefore, at least a partial expression of the pp60<sup>re</sup> molecule on the outer cell surface cannot yet be excluded.

Establishment of hybridoma cell lines secreting antibodies against pp60<sup>re</sup> and/or TSSA can easily be monitored using the PKA test and immunofluorescence, respectively. Corresponding monoclonal antibodies should help to reveal possible antigenic and biochemical relationships between the two transformation-specific proteins.

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Friedrich-Miescher-Laboratorium
Max-Planck-Institute
7400 Tübingen,
West Germany

ADOLFINE HUESGEN
GERT HUESGEN
JOHANNES STEGMANN
REINHARD KURTH

REFERENCES


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