Immunological Characterization of a Mammary Tumour Virus from Swiss Mice: Multiple Epitopes Associated with the Viral Gene Products

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SUMMARY

The major antigens of a mouse mammary tumour virus (MMTV) isolated from the milk of exogenously infected MB+ Swiss mice were compared with the viral components of other MMTV strains by using the methodology developed by Teramoto et al. (1977a). The anti-gp47 (Swiss) serum differentiated between type- and group-specific antigenic determinants on the major glycoprotein; two distinct type-reactivities were demonstrated, one of them being shared by the MMTVs (Swiss) and (RIII), and the other by the MMTVs (C3H) and (GR). The MMTVs (Swiss) and (RIII) also reacted identically in a ‘type-specific’ assay using an anti-p28 (Swiss) serum, but a distinct reactivity was observed with the MMTV (C3H). In the isologous test where an anti-(total RIII) serum was used to bind intact, externally labelled RIII virions, the Swiss virus was seen to possess a type-specific determinant on its surface which distinguished itself from both MMTVs (RIII) and (C3H). The Swiss/fC57BL mice (which are devoid of the milk virus and for this reason are referred to as MB−), expressed only the internal virus antigens in their mammary glands. Under the ‘type-specific’ assay conditions, the p28 antigen present in the mammary gland extracts of the MB+ mice was indistinguishable from the p28 antigen purified from the Swiss virion, but was clearly distinct from the p28 reactivity present in the mammary gland extracts of the MB− mice. The p28 (MB−) antigen may thus represent the expression of an endogenous virus sequence different from the milk virus genome.

INTRODUCTION

Swiss albino mice from a closed colony, randomly bred in our laboratory since 1955, produce mammary tumour viruses (MMTV) in high amounts in their milk (Calberg-Bacq et al., 1976). Inbreeding these mice started in 1978; since enough generations have not been obtained to allow genetic analysis, the line has been called MB+ (for the presence of the milk-borne virus). The mammary tumour incidence among these mice is moderately high: 10 to 30% for 6- to 12-month-old breeding females. Swiss mice are generally regarded as low tumour incidence strains (Toth & Shimizu, 1974; Staats, 1980) except for the lines SHN and SLN (inbred in Japan), which were selected for a high incidence of mammary tumours appearing early and late in the mouse life respectively (Nagasawa et al., 1976; Imai et al., 1980). We have also developed an MB− line by foster-nursing MB+ mice on C57BL mice. The MB− Swiss mice neither produce milk viruses nor have so far presented any mammary tumours; they represent a clear example of non-coordinate virus expression since high
amounts of p28 antigen are present in the mammary glands of the adult female, while the antigen gp47 cannot be detected (Osterrieth et al., 1979; Kozma et al., 1979). Such a partial expression of MMTV is also characteristically observed in the mammary glands of the GR/MTV-2- mice (Nusse et al., 1980), and in cultures of mammary tumours and preneoplastic mammary tissues from Balb/c mice (Teramoto et al., 1980).

MMTVs are closely related viruses; they possess common group-specific determinants that can be estimated by radioimmunoassays using specific antisera at the highest possible dilutions (Zangerle et al., 1977; Hendrick et al., 1978). However, low dilutions of the antiserum (i.e. when the competition is measured for a large population of antibodies), permits detection of type-specific determinants (Teramoto et al., 1977a). On the basis of the distinct reactivities that occur on the major envelope glycoproteins gp52 and gp36 (Teramoto et al., 1977a; Teramoto & Schlom, 1979) and on the major internal polypeptide p28 (Teramoto & Schlom, 1978), the C3H, RIII and GR viral strains have been shown to represent different types of MMTVs. A type-specific assay based upon gp52 reactivities also permitted differentiation between the exogenous, highly oncogenic and the endogenous, weakly oncogenic MMTVs that are expressed in the same mouse strain (Teramoto et al., 1977b; Arthur & Fine, 1979; Massey et al., 1980). Further evidence for the existence of distinct type-specific reactivities has been provided by tryptic peptide analyses (through which various gp52s, p28s and p10s have been identified; Gautsch et al., 1978) and more recently, by the use of monoclonal antibodies which recognize individual epitopes on the viral gp52 antigens (Massey et al., 1980).

Since both the type of the virus itself and the mouse genotype are important parameters in the processing of an MMTV infection, experiments were carried out to further characterize our Swiss mouse mammary tumour system. The present study deals with the immunological typing of some of the virus gene products, i.e. the main internal protein (p28), the main external glycoprotein (gp47; in our gels the estimated mol. wt. for this major component is 47 000) and determinants borne by the surface of the intact virion. This study also provides a characterization of the p28 reactivities that are exhibited by the mammary gland cells of the MB+ and MB- mice respectively.

**METHODS**

**Viruses.** MMTV (Swiss) was purified from the milk of Swiss MB+ mice as described by Calberg-Bacq et al. (1976), except for the presence of a protease inhibitor in the TNE buffer (10 mM-tris–HCl buffer pH 7.2, containing 0.1 M-NaCl, 30 mM-EDTA and 0.04% phenylmethylsulphonyl fluoride). The following viruses were prepared as described by Teramoto & Schlom (1979): MMTV (RIII) from the milk of multiparous RIII mice (Meloy Laboratories, Springfield, Va., U.S.A.); MMTV (C3H) from the culture fluids of the Mm5mt/C1 mammary tumour cell line; MMTV (GR) from culture fluids of a GR mammary tumour cell line; MMTVs (C3H)Fe and (RIII)Fe from the corresponding culture fluids of infected feline kidney cells.

**Protein determination.** The method of Lowry et al. (1951) was used with bovine serum albumin as standard.

**Antigen p28 (Swiss) preparations from lactating mammary glands.** Cellular p28 antigens were prepared from mammary glands (around 27 g) of lactating MB+ and MB- Swiss mice respectively. Minced tissues were incubated for 1 h at 4 °C in the presence of Trasylol (Bayer, 1000 U/ml), phenylmethylsulphonyl fluoride (0.6 mg/ml) and 30 mM-EDTA (final concentrations), were homogenized and extracted with 3 M-KCl (final concentration) in 50 mM-phosphate buffer pH 6.4. After incubation at 4 °C for 15 h, the extracts were extensively dialysed against phosphate buffer and clarified by centrifugation at 1000 g for 20 min. Precipitation with (NH4)2SO4 permitted recovery of 90% of the p28 reactivity in those
fractions collected between 35 and 55% saturation. The precipitates thus obtained were resuspended in 50 mM-phosphate buffer pH 6-4 containing 0.45 M-NaCl and the suspensions clarified by centrifugation at 10000 g. On the basis of the immunoreactivity, the final yield was 240 μg of Swiss MB+ p28 and 200 μg of Swiss MB− p28.

**Purified antigens and specific antisera.** Glycoprotein gp47 (Swiss) and protein p28 (Swiss) were prepared from Swiss milk particles (Zangerle et al., 1977; Hendrick et al., 1978). Specific antisera against purified gp47 (Swiss) and against purified p28 (Swiss) were raised in rabbits (Hendrick et al., 1978). Antisera against total RIII virions and total C3H virions were prepared in rabbits as described by Teramoto et al. (1977a).

**Labelling with 125I.** The purified gp47 (Swiss) and p28 (Swiss) antigens (20 μg dissolved in 200 μl TNE buffer, 10 mM-tris–HCl buffer pH 7.2 containing 0.1 M-NaCl and 1 mM-EDTA) were iodinated by using the Iodogen method (Devare & Stephenson, 1977). After filtration on a Sephadex G25 column (0.8 × 24 cm), the radioactive samples were extensively dialysed against the TNE buffer supplemented with 5 mM-NaI. Purified RIII virions (100 μg) were externally labelled by the same technique and the intact, iodinated virions were recovered following isopycnic banding in a sucrose gradient. The specific radioactivities were 4 × 10⁶, 1.5 × 10⁶ and 0.5 × 10⁶ ct/min/μg protein, for gp47 (Swiss), p28 (Swiss) and MMTV (RIII) virions respectively. Purified Swiss virions became unstable when iodinated by this technique. Polyacrylamide gel electrophoreses of the iodinated Swiss gp47 and p28 antigens in the presence of SDS and urea (Teramoto et al., 1977a) revealed one single protein band in each case. In contrast to the results obtained by the lactoperoxidase iodination (Teramoto et al., 1977a), the Iodogen method resulted in the labelling of several external components of the RIII virion (gp36, gp52 and higher mol. wt. glycoproteins).

**Titration of the antisera.** The antisera were titrated following the technique described by Teramoto et al. (1977b). With the purified antigens, the dilutions were made in the TNE buffer supplemented with 0.1% Triton X-100 and 0.2% (w/v) serum albumin. With the intact virions, a borate–saline buffer (0.13 M-borate–0.13 M-NaCl adjusted to pH 7.2), supplemented with 0.2% serum albumin, was used. The titres of the various sera, expressed as the dilution which precipitated 25% of the isologous 125I-labelled antigens were 1:40000 for the anti-gp47 (Swiss) serum, 1:560000 for the anti-p28 (Swiss) serum and 1:400000 for the antiserum raised against RIII virions.

**Competition radioimmunoassays.** The tests were performed as described by Teramoto & Schlom (1978) in TNE buffer supplemented with 0.1% Triton X-100 and 0.2% serum albumin. The competitors, previously treated with NP40 (0.6% final concentration), were added to a limited amount of antiserum and the solutions incubated for 1 h at 37 °C, after which time the labelled antigens, either gp47 (Swiss) or p28 (Swiss) were added (10000 ct/min) and the reaction mixtures incubated for another hour at 37 °C. Finally, the samples were supplemented with anti-(rabbit IgG) serum and were successively incubated for 15 h at 37 °C and for 15 h at 4 °C. After centrifugation at 10000 g for 1.5 min, the supernatants were removed and the pellets assayed for radioactivity. When intact virions were the labelled antigens, the NP40 treatment was omitted and the borate–saline buffer was used as diluent. Washing the pellets did not modify the radioactivity measurements.

**RESULTS**

**Type- and group-specific reactivities on the gp47 (Swiss) antigen**

Isologous assays were used to measure the abilities of the MMTVs (Swiss), (RIII), (C3H) and (GR) to inhibit the binding of 125I-labelled gp47 (Swiss) to the anti-gp47 (Swiss) serum. Group-specific assays carried out at a high dilution of the antiserum (1:40000), showed that the various MMTVs had similar competing efficiencies. Addition of 20 ng of either RIII or
Swiss viruses inhibited the precipitation reaction by 50%. The same extent of competition was observed with even lower amounts of the two other MMTVs (Fig. 1b). In turn, type-specific assays carried out at a very low antibody dilution (1:500) gave rise, depending upon the MMTV used as competitor, to different competition patterns (Fig. 1a). Both MMTVs (Swiss) and (RIII) on the one hand, behaved identically; the linear portions of the competition curves exhibited the same slope and at high concentrations the two MMTVs inhibited the precipitation of the labelled antigen by more than 90%. Both MMTVs (C3H) and (GR) on the other hand, had a much decreased competing efficacy; the slope of the curve was diminished and the inhibition of the precipitation reaction did not reach completion.

Because the competing viruses had various origins [MMTVs (RIII) and (Swiss) were from milk samples; MMTVs (C3H) and (GR) were from culture fluids], the question arose whether or not the observed difference might be due to a milk component. Assays at the same high and low dilutions of the anti-gp47 (Swiss) serum as above were therefore performed using as competitors MMTVs (RIII)Fe and (C3H)Fe grown in the same feline cell line. The anti-gp47 (Swiss) serum differentiated between these two viruses in the type-specific reaction (Fig. 1c), but an identical competition pattern was observed in the group-specific reaction (data not shown). The high specificity of the reaction was also confirmed by the fact that as much as 20 μg of either type C or type D retroviruses, 100 μg of milk or gland extract from uninfected mice and 100 μg of foetal calf serum proteins were completely devoid of any competing activity (Fig. 1c).

Type- and group-specific reactivities on the p28 (Swiss) antigen

In the assays described below, the anti-p28 (Swiss) serum was always used at the low 1:500 dilution. The type-specific reaction was an isologous Swiss assay in which competition for binding of the radioactively labelled p28 (Swiss) was measured. The group-specific assay was a homologous test carried out with the same population of highly concentrated antibodies as in the type-specific reaction except that the bound, labelled antigen was p28 (C3H), prepared as described by Teramoto & Schlom (1978). Under type-specific conditions (Fig. 2a), the competition due to increasing amounts of MMTV (C3H) differed from that observed with both MMTVs (Swiss) and (RIII). These two viruses behaved identically and,
Multiple epitopes on MMTV (Swiss) antigens

Fig. 2. Type- and group-specific reactivities on p28 (Swiss). Anti-p28 (Swiss) serum was used at a final dilution of 1:500 to bind either $^{125}$I-labelled p28 (Swiss) in (a) and (c) or $^{125}$I-labelled p28 (C3H) in (b) and (d). In (a) and (b) the competitors were MMTVs (Swiss) (x), (RIII) (△) and (C3H) (○). The controls were the same as those of Fig. 1 (c). In (c) and (d) the competitors were MMTVs (C3H)Fe (●) and (RIII)Fe (△), grown in feline cells.

when present in high amounts, they inhibited the $^{125}$I-labelled p28 (Swiss) antigen precipitation by more than 80%. Competition due to C3H virions was less efficient: the resulting graph presented a reduced slope. Using the same isologous Swiss assay, the MMTV (C3H)Fe was also clearly distinct from the MMTV (RIII)Fe (Fig. 2c). Other C or D retroviruses and milk or cell extracts from uninfected mice had no competing activity (Fig. 2a).

In the group-specific assay based on the binding of $^{125}$I-labelled p28 (C3H) by the anti-p28 (Swiss) serum, the same amount of p28 reactivity was titrated in the C3H, RIII and Swiss viruses (Fig. 2b) and, when used in high amounts, each of these three viruses inhibited the precipitation reaction by at least 90%. The same group-specific assay gave superimposable competition curves with both MMTVs (C3H)Fe and (RIII)Fe (Fig. 2d). An isologous C3H test was also performed using the system anti-(total C3H) serum (at a 1:1000 dilution) and $^{125}$I-labelled p28 (C3H) antigen (Teramoto & Schlom, 1978). Those preparations of MMTVs (Swiss), (RIII) and (C3H), which gave rise to identical reactions in the group-specific assay described above, were used as competitors. High amounts of MMTV (C3H) caused a total inhibition of the labelled antigen precipitation reaction, whereas competition by either MMTVs (RIII) or (Swiss) was incomplete, and the linear portions of the corresponding competition curves presented altered slopes (data not shown).

Type- and group-specific reactivities on the surface of the Swiss virion

The type-specific assays described above failed to differentiate between Swiss and RIII viruses with regard to the gp47 and p28 antigens. Another assay was therefore devised to investigate whether or not distinct immunological reactivities were present at the surface of these two virions. Preliminary experiments were carried out to determine the conditions under which the competition slopes due to the MMTVs (RIII) and (C3H) were maximally different in the [anti-(total RIII) serum + $^{125}$I-labelled intact RIII virions] isologous system. The anti-(total RIII) serum finally selected was used at a 1:2000 dilution (see legend of Fig. 3a); under these conditions, RIII and C3H virions (50 μg) were found to inhibit binding of the radioactively labelled RIII virions by 84% and (at the most) 20% respectively. The MMTV (Swiss) was then used in the same test; as shown in Fig. 3(a), its competing ability was intermediate to those of the MMTVs (RIII) and (C3H). Note that when the group-specific reactivities were titrated in parallel, the three virus suspensions gave rise to identical competition curves (Fig. 3b).
Immunological differentiation between the p28 antigens isolated from mammary glands of \(MB^+\) and \(MB^-\) Swiss mice

The same type-specific assay conditions as those described previously to distinguish p28 reactivities were used to investigate whether or not the p28 (Swiss) antigen, which occurs in...
Multiple epitopes on MMTV (Swiss) antigens

the mammary glands of the MB− mice (which are devoid of the milk virus), was the same as the p28 (Swiss) antigen which occurs in the mammary glands of exogenously infected MB+ mice. In such tissues, the p28 antigen expression has been previously determined (per mg of total proteins, 275 ng p28 and 100 ng p28 were found in MB+ and MB− glands respectively). Cellular extracts enriched in p28 antigen were prepared from mammary glands of both MB+ and MB− mice at late pregnancy (first to third parity) and the amounts of the p28 antigen were measured by the usual radioimmunoassays at high antiserum dilution. On the basis of the data thus obtained, the group-specific assay showed that increasing amounts of p28 antigen had identical competing efficiencies whether the antigen originated from an MB− cellular extract, from an MB+ cellular extract or from the Swiss milk virus itself (Fig. 4 b). However, when the same isologous test was performed at a low 1:500 serum dilution (Fig. 4 a) two observations were made: (i) the cellular MB+ p28 and the viral p28 (Swiss) were competitors of equal efficiency, both of them causing, at high concentrations, a complete inhibition of the binding of the labelled antigen; (ii) when present in similar high amounts, the cellular MB− p28 antigen, was a weaker competitor and the resulting graph exhibited a very reduced slope.

Two additional observations (data not shown) confirmed the results presented in Fig. 4 (a): (i) the cellular MB− p28 and MB+ p28 antigens still behaved differently when the anti-p28 (Swiss) serum was used at a higher dilution of 1:1000; (ii) after partial absorption of the anti-p28 (Swiss) serum by the MB− cellular extract (incubation at 37 °C for 1 h and then at 4 °C for 15 h followed by 30 min centrifugation at 10000 g), the radioimmunoassay revealed that the cellular MB− p28 antigen has a reduced competing ability even when the test was performed at a serum dilution of 1:10000, thus indicating that the selective absorption of antibodies by the MB− p28 antigen has resulted in an amplification of the type-specific response.

DISCUSSION

Type-specific radioimmunoassays have been developed which make use of highly specific antisera directed against purified antigens from an MMTV secreted in the milk of Swiss mice. On the basis of both their gp47 and p28 reactivities, the C3H and RIII virions appear to be significantly distinct from each other, the slope differences measured in the Swiss assays being as large as those measured in the C3H or RIII isologous tests by Teramoto et al. (1977 a) and Teramoto & Schlom (1978). When compared with the gp47 reactivity, the p28 reactivity exhibits a less pronounced type-specific response. In both gp47 and p28 type-specific assays, the MMTVs (Swiss) and (RIII) react identically but these two viruses can be distinguished from each other in a test which is directed towards the entire surface of the virions. A likely explanation is that an immunoreactivity associated with a specific epitope occurs on the virus-coded envelope glycoprotein gp36 (which is the viral antigen exhibiting the most pronounced type-specificity; Teramoto & Schlom, 1979). However, this antigenic difference might also be due to host components that would be incorporated in (or bound to) the virus envelope (although such components are found in very low amounts and are probably more organ-specific than mouse strain-specific).

The reactivity of the cellular p28 antigen from the MB+ mice is identical to that of the p28 antigen purified from the virus. Very strikingly, however, the type-specific assay reveals large differences between the p28 antigen that is expressed from the exogenous Swiss virus and the p28 antigen that is expressed from viral sequences endogenous to the Swiss mice. Not only are these two antigens immunologically distinct, but other studies have shown that their syntheses in the mammary glands can be morphologically distinguished by immunofluorescence (Kozma et al., 1979) and electron microscopy (C-M. Calberg-Bacq et al., unpublished results). At this time, the C3Hf virion has been differentiated at the level of the
gp52 antigen from the exogenous MMTVs, especially the MMTV (C3H), by type-specific radioimmunoassay using anti-(total virus) sera raised in rabbits (Teramoto et al., 1977b), infected mice sera (Arthur & Fine, 1979) and monoclonal antibodies (Massey et al., 1980). The fact that virions are not produced in foster-nursed mice demonstrates that the virus expression in the MB- mice is different from the virus expression in RIII or C3Hf mice. Recently, it has been shown that the production of abnormal virus particles by some lymphoma cell lines is due to a blockage of the processing of the 'env' polypeptide precursor (Nusse et al., 1979; Vaidya et al., 1980). A similar situation does not apply to the MB- mice since any gp47 reactivity cannot be detected in the mammary gland cells (Osterrieth et al., 1979). The presence of p8 (p10) antigen, however, has been demonstrated in the MB- mammary glands by immunofluorescence (Kozma et al., 1979), indicating that the complete precursor of the internal peptides is probably synthesized. Both p10 and p28 antigens but none of the 'env' glycoproteins, are also found in the mammary glands of GR/MTV-2- mice, a substrain congenic to the GR but lacking the MTV-2 gene for high virus expression and early mammary tumours (Nusse et al., 1980). Situations as those described for MB- and GR/MTV-2- might be caused by MMTV proviral sequences that would be defective in the 'env' gene itself, or alternatively in the transcription or the processing of the subgenomic mRNA coding for the virus glycoproteins (Robertson & Varmus, 1979). Whether or not the endogenous 'gag' gene expression plays a role during the productive infection with the milk virus is unknown. The antigenic differences between the two p28 antigens that have been described above offers a possibility to study this problem in the MB+ system.

In conclusion, the occurrence of various epitopes on the MMTV (Swiss) virus gene products shows that the Swiss exogenous virus might be distinct from, although closely related to, the MMTV (RIII). Virus sequences that are endogenous in the Swiss mice and are partially expressed in the MB- mammary cells, give rise to a p28 antigen which is largely different from the corresponding component in the exogenous virus. With regard to the origin of the MMTV (Swiss), the results obtained at this time do not favour an accidental expression of endogenous sequences. Rather, they support the view that an accidental infection (horizontal?) by RIII or an RIII-related virus has caused in the Swiss mice a much lower incidence of early tumours than that observed in the RIII mice.

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