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VIRAL GLYCOPROTEINS SYNTHESIS IN FRIEND CELL LINES PRODUCING POLYCYTHEMIC (FV-P) AND ANEMIC (FV-A) FRIEND VIRUSES.

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SUMMARY

Two tumor Friend cell lines producing anemia-inducing virus (TF-A line) or polycythemia-inducing virus (TF-P line) were compared for their viral-encoded glycoproteins. The envelope glycoproteins of the two viral populations differ by their electrophoretic mobilities. The gpr^{env} precursors also differ by their relative mobilities. The TF-P cells contain the typical gp50-52 molecular species, which is coded for by Spleen Focus Forming sequences (SFFV) present in the genome of the polycythemia-inducing virus. The TF-A cells do not contain the gp50-52, but express in small amounts a species with a higher apparent molecular weight. This species which has been named FV-A gp55 could be equivalent to the gp50-52 coded for by the SFFV sequences. Very similar results were obtained with leukemic cells prepared from enlarged spleens of mice infected with the anemic or polycythemic Friend viruses.

INTRODUCTION

There are at least two strains of leukemogenic Friend virus. The original stock isolated in 1957 by C. Friend induces an anemia (FV-A), while the polycythemia-inducing virus (FV-P), which contains the Spleen Focus Forming Virus genome (SFFV) provokes an erythropoietin-independent erythropoiesis followed by a polycythemic state (See the recent review by Tambourin et al., 1). Macroscopic foci in the spleen of susceptible mice have been related to SFFV, while FV-A is reputed not to give the same effect (2). Recently, molecular studies conducted with FV-P have demonstrated that the SFFV genome shares some homology with its helper Fr-MuLV and contains some specific sequences coding for a glycoprotein gp50-52, which partially cross-reacts with Rauscher gp70, the envelope glycoprotein of murine C-type retroviruses (3, 4, 5). This gp50-52 glycoprotein is absent from virions and has been postulated to be implicated in the pathological potential of SFFV (4, 5). Hence, it was interesting to look at its possible presence in FV-A producing erythroblastic cells.

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In this work, the viral-encoded glycoproteins of cells producing respectively FV-A and FV-P were compared by electrophoresis on polyacrylamide gels. Two main differences were found : the FV-P-producing erythroblastic cells contain a typical gp50-52 molecular species, whereas FV-A-producing erythroblastic cells express in low amounts a species with a slightly higher apparent molecular weight (55 K). On the other hand, the envelope glycoproteins of the respective helper viruses of FV-A and FV-P are different in their electrophoretic mobilities.

The same type of results were observed with spleen cells from leukemic Friend mice.

MATERIAL AND METHODS

i) Reagents

- Cell lysis buffer : 0.05M Tris-HCl, pH 7.5 ; Sodium Deoxycholate, 0.5 % ; Sodium Dodecylsulfate 0.1 % ; 0.25M NaCl ; 0.001M EDTA.
- ^{3}H leucine, ^{3}H valine. Commissariat à l'Energie Atomique, Saclay, France. Specific activities : 35 Ci/mM and 13 Ci/mM respectively.
- Phenylmethylsulfonyl Fluoride (PMSF), Boehringer.
- Staphylococcus aureus Cowan Strain I : Pasteur Institute, Paris.
- Goat anti gp70 Batch 78S-22S, anti serum was obtained from the National Cancer Institute through the Special Virus Cancer Program. It was prepared using purified Rauscher virus stocks grown in cell cultures.
- Kodak X-O MatXR medical films were used for fluorography.

ii) Origin of FV-A and FV-P viruses

The anemia-inducing Friend virus (FV-A), originally obtained in 1958 from Dr Charlotte Friend, was maintained in the Institut Curie, Paris-Orsay. It was purified by the spleen clone method of Pluznick and Sachs (6) and has undergone over 60 cell-free passages in Swiss mice of our colony. It was then adapted in DBA/2 mice by 4 successive cell-free passages. It induces an erythroleukemia associated with anemia, as did the original isolate (7).

The polycythemia-inducing Friend virus (FV-P) was obtained from Dr H. Yoshikura (8) and has gone through 8 cell-free passages in DBA/2 mice. It contains the defective spleen-focus-forming (SFFV) and induces an erythroleukemia associated with polycythemia.

For the present studies, virus stocks were prepared from a pool of leukemic spleens, stored in liquid nitrogen. Spleens were homogeneized in Minimum Essential Medium (MEM) (1 g/10 ml). The homogenates were centrifuged for 20 minutes at 10,700 g and the supernatant collected and filtered through a 0.45 µm Millipore membrane. For use, the samples were diluted ten fold in MEM. The virus suspensions were inoculated intravenously via the retro orbital sinus of mice. Both stocks of virus are N-tropic.

iii) Cells

Tumor cell cultures

Tumorigenic cells were isolated from enlarged spleens of DBA/2 MRC donor mice injected 40 days previously with FV-P or 90 days previously with FV-A by subcutaneous graft. Tumor cell lines which were established in culture were called TF-A11 and TF-P10 cell lines (for Tumor Friend Anemia and Tumor Friend Polycythemia). Culture conditions were in non agitated suspensions in Mac Coy or RPMI 1629 media supplemented with 15 % heat-inactivated

fetal calf serum, 5mM glutamine and without antibiotics. The cells were passed twice or three times a week and were at their 30th passage at the time of the experiments. More details on the characteristics of these two cell lines will be described elsewhere.

Leukemic spleen cells

DBA/2 mice were intravenously inoculated with FV-A of FV-P viruses. On the 30th day for FV-A and the 14th day for FV-P, spleens weighing 1,400 and 1,600 mg respectively were teared into small pieces. Cell suspensions were washed twice in Minimum Essential Medium (MEM) without valine and leucine and resuspended at a 5.10^6 cell/ml concentration in the labelling medium (see the labelling conditions). 4.10^8 cells were used per experiment.

Friend-Eveline cells

This fibroblastic cell line originally infected with the Friend virus complex produces only lymphatic leukemia virus (9). It was grown in suspension in MEM plus 10 % heat inactivated fetal calf serum and 10 % Tryptose phosphate (10).

iv) Labelling of cells

Exponentially growing cultures were pooled by centrifugation and suspended at a final concentration of 2 to 4.10^6 cells/ml in MEM without leucine and valine and containing 10 % heat inactivated non dialyzed fetal calf serum. Sixty minutes later, 150 µCi/ml of ^3H leucine were added and the cultures were incubated for 20 minutes at 37°C. At the end of this period, cells were centrifuged at 1,500 rpm for 5 minutes. One half of the culture was used for preparing a cell extract and the rest was suspended at 2.10^5 cells/ml in normal MEM. Radioactivity was chased by maintaining the cells for 4 hours at 37°C.

Labelling of the leukemic spleen cells was performed in the same way with the difference that it was continued for 4 hours.

v) Preparation of cellular extracts

Cells were washed twice in Phosphate Saline Buffer (PBS) and homogenized at 0°C in lysis Buffer (3 volumes). PMSF was extemporaneously added to the lysis Buffer (0.001M). After 15 minutes at 0°C, cell extracts were centrifuged for 5 minutes at 2,000 rpm and 15 minutes at 10,000 rpm. Supernatants were directly used for immunoprecipitation.

vi) Immunoprecipitation

Usually, $1-2.10^6$ cpm were employed to perform each immune reaction in a 0.5 ml final volume of cell lysis Buffer. The anti gp70 antiserum (2 to 4 µl) was added and the mixtures were incubated for two hours at 37°C and then, overnight at 4°C. The antigen-antibody complex was precipitated by addition of 100 µl of a 10 % Formaline-inactivated Staphylococcus aureus suspension (Cowan 1 strain) prepared according to Kessler (11).

vii) Protein analysis

Electrophoresis was performed in 5-20 % polyacrylamide slab gels for 17 hours at 5.5 volts/cm using Laemmli's conditions (12). Gels were processed for fluorography according to Bonner and Laskey (13).

RESULTS

1) Biological properties of the viruses produced by TF-A11 and TF-P10 cell lines

Supernatants of TF-A and TF-P cell cultures were taken at different passages, filtered through 0.45 µm Millipore membranes and iv. inoculated into

young adult DBA/2 mice. In both cases, an erythroleukemia developed in few weeks following inoculation. Examination of the blood of the animals revealed an anemia on the 21th day in the case of TF-A11 culture supernatants and a polycythemia, on the 14th day in the case of TF-P10 supernatants, values of the hematocrite being respectively 30 and 75 %. When the spleens of the animals were examined on day nine after inoculation of a previously determined dilution of the cell supernatants, those resulting from TF-P10 culture supernatant were characteristic of the SFFV Friend virus component, whereas those induced by TF-A11 culture supernatants had a more discrete aspect.

These facts demonstrate that the viruses produced by TF-A11 and TF-P10 erythroleukemic cells retained their original pathogenic potential.

2) Glycoprotein composition of the viruses released by TF-A11 and TF-P10 cell lines

Since the Fr-MuLV component of the Friend virus complex provides the envelope glycoprotein (gp70), we examined comparatively the glycoprotein content of purified virus isolated from TF-A11, TF-P10 cells and also from Friend Eveline cultures which served as a reference. Equivalent amounts of ^{3}H-amino acid labelled TF-A11 and TF-P10 virus were co-migrated with Friend-Eveline virus in a 5-20 % polyacrylamide slab gel. Glycoproteins were detected by their mobility and their capacity to be specifically precipitated by a goat antiserum directed against Rauscher gp70. The pattern on the figure 1 clearly shows that the glycoprotein of FV-A and FV-P virus do not move at the same position in the gel. More precisely, if a molecular weight value of 70,000 is taken as a reference for gp70 of the Friend-Eveline virus (wells e and f), the apparent molecular weight of FV-A and FV-P glycoproteins can be estimated respectively to 65-66,000 (wells a and b) and 72-73,000 (wells c and d). larly in the 50-55 K region. This confirms that the gp50-52 molecular species of erythroleukemia Friend cells is not detectable as a structural viral protein (3, 4, 5 and see the following section).

3) Analysis of viral proteins in TF-A11 and TF-P10 cell lines

a) Viral envelope glycoproteins

An approach similar to the one used in the previous section was performed to compare the biosynthesis of the envelope glycoproteins in TF-A, TF-P and Friend-Eveline cells. Cultures were pulse-labelled for 20 minutes with ^{3}H leucine and valine, then, chased for several hours in cold medium. Cellular extracts were prepared as described in materials and methods and immunoprecipitated with anti Rauscher gp70 goat antiserum. The figure 2 presents the results. At 20 minutes, there is only one major band corresponding to the envelope glycoprotein precursor (gpr^{env}) but its mobility differs in each cell

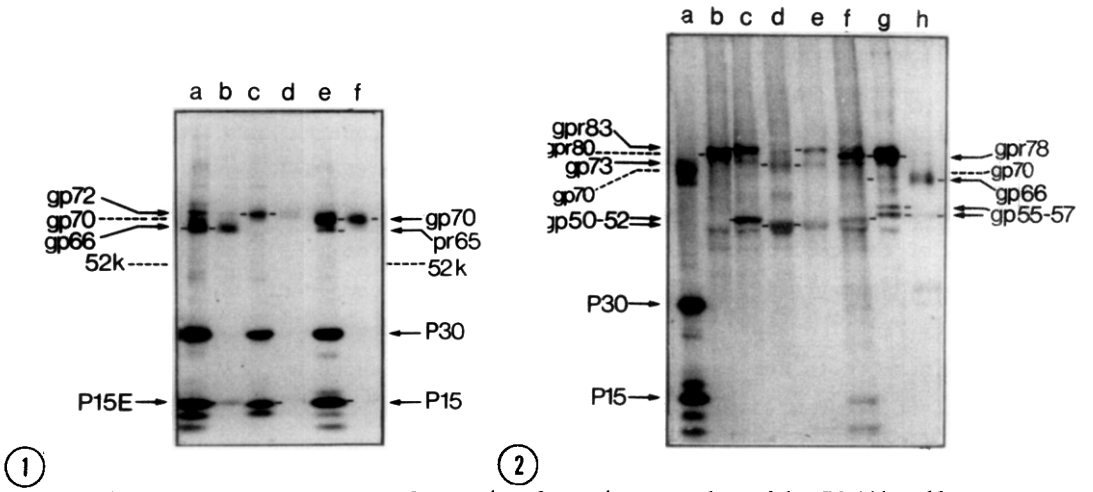

Fig. 1 : Electrophoregrams of proteins from viruses released by TF-A11 cell cultures, TF-P10 cell cultures and Friend-Eveline cell cultures.

Cell cultures were labelled for 24 hours with ^{3}H leucine and ^{3}H valine (2.5 µC/ml). Viruses were isolated from the supernatants by sedimentation and equilibrium sucrose gradient centrifugation (10). Samples were directly loaded onto the gels or dissociated with lysis buffer and immuno-precipitated by anti gp70 Rauscher anti-serum.

a : TF-A11 virus total proteins.
b : the same immuno-precipitated by anti gp70 Rauscher anti-serum.
c : TF-P10 virus total proteins.
d : the same immuno-precipitated by anti gp70 anti-serum.
e : Friend-Eveline virus total proteins.
f : the same immuno-precipitated by anti gp70 anti-serum.

Fig. 2 : Analysis of the glycoproteins precipitated by anti gp70 Rauscher antiserum in cellular extracts of TF-A11, TF-P10 and Friend-Eveline cells in culture and leukemic spleen DBA/2 mouse cells.

a : Friend-Eveline virus total proteins runned as markers.
b : Friend-Eveline cell extract labelled for 20 minutes.
c : TF-P10 cell extract labelled for 20 minutes.
d : TF-P10 cell extract labelled for 20 minutes and chased for 4hours
e : Leukemic cell extract from the spleen of a DBA/2 mouse inoculated with FV-P. Cells were labelled for 4 hours.
f : Leukemic cell extract from the spleen of a DBA/2 mouse inoculated with FV-A. Same labelling time as in e.
g : TF-A11 cell extract labelled for 20 minutes.
h : TF-A11 cell extract labelled for 20 minutes and chased for 4 hours.

line. The apparent molecular weights of each gpr^{env} molecular species were estimated to be 80,000 for Friend-Eveline cells (well b), 78,000 for TF-A cells (well g) and 83,000 for TF-P cells (well c). Besides this major material, fainter bands are visible in the TF-P10 extracts, which move in the 78-80,000 region. After a 4 hours chase, most of the radioactivity of the gpr^{env} molecules have disappeared and are redistributed into bands moving at the same position as the viral glycoproteins precedently described. These results indicate that the apparent size differences found in the virus glycoproteins also exist in their precursors, suggesting that the "env" gene information differ in the particles of the TF-A and TF-P virus populations.

b) Rauscher gp70-related molecules in the 50-55K region

Antibodies directly against the Rauscher envelope murine glycoprotein have been reported to precipitate a 50-52,000 glycoprotein in the Friend tumor cell lines (3,4). Examination of the electrophoretic patterns presented in figure 2 indicates that it was indeed the case for TF-P cells (wells c and d). At 20 minutes labelling (well c), a proeminent band composed of material with an apparent molecular weight of 52,000 occurs in the gel. During the 4 hours chase (well d), this band slightly shifts and its molecular weight was estimated to be 50-52,000. Moreover, its intensity is greater that that of the $gpr83^{env}$ and gp73. This result corroborates perfectly those of the literature.

For the TF-A11 cells, the situation is somewhat different (wells g and h). At 20 minutes, no major band equivalent to gp50-52 FV-P is present in the gel but rather two fainter running more slowly in the 57K regions indicated by arrows in well g. After 4 hours of chase in cold MEM, these two faint bands have disappeared and are replaced by a broader material in the region of 55K (well h). This result was reproducibly observed and we concluded that TF-A11 cells express at a lower rate, a gp55 species differing from the gp50-52 of FV-P by its mobility.

The same analysis was done with Friend-Eveline cells, which produced only lymphatic leukemia virus (Fr-MuLV). No material was precipitated by anti gp70 serum in the 55K region (well b).

4) Analysis of Rauscher gp70-related glycoproteins in DBA/2 mouse leukemic spleen cells

In order to ascertain that our results were not due to the use of established cell lines, experiments were repeated using cells isolated from leukemic spleen cells of DBA/2 mice previously injected with FV-A or FV-P inoculum (see material and methods). The results of the figure 2 corroborate those described precedently. Particularly, one can observe a difference in the mobilities and the intensities of the materials running in the 50-55K region of the gel. It can be noticed that the spleen cells of DBA/2 mice have been labelled for 4 hours. At this time, the glycoproteins have achieved their processing and run in the gels at the same position as the gp70-related molecules synthesized by TF-P10 and TF-A11 tumor cells during a 20 mn pulse followed by a 4 hours chase (compare wells 2e and 2d and 2f and 2h). It can be also noticed that the behaviour of the gpr^{env} and envelope glycoproteins is identical to that of the viruses produced by TF-A11 and TF-P10 cell lines (wells e and f).

RNA expression of the SFFV genome has been reported in normal hemopoietic cells of uninfected adult mice (14). Experiments were performed with nor-

mal spleens of adult DBA/2 mice previously bled for 3 days in order to accelerate the erythropoietic process. No Rauscher gp70-related product was detected in cellular extracts treated in the same conditions as leukemic spleen cells (not shown). These negative results may be only due to the relative lack of sensitivity of our experimental conditions.

DISCUSSION

The viruses released by TF-A and TF-P cells differ by their envelope glycoproteins, whose apparent molecular weights are 66,000 and 73,000 respectively. These molecules possess at least a common antigenic site, since both are precipitated by an antiserum against Rauscher gp70. To date, it is impossible to say wether these differences are important in view of the role these viruses can play during the establishement of the leukemic disease.

The presence of a gp50-52 in various erythroleukemic and fibroblastic cells containing the SFFV genome is well documented (3, 4, 5). We have confirmed this result with an established erythroblastoid tumoral cell line as well as with spleen leukemic cells from mice precedently injected with FV-P. This glycoprotein has the properties reported by others i.e., it is apparently only present in cells and is processed soon after its synthesis as shown by the increase in its electrophoretic mobility. The origin of this change is not precisely known but is likely to be the consequence of several steps in the glycosylation of the molecule (15).

We have found that FV-A infected cells do not contain the same gp50-52 molecular species. However, examination of this region of the gels reveals the presence of material which could be equivalent. The electrophoretic position of this material after a 20 minutes pulse is compatible with a 57K apparent molecular weight but is shifted at a 55K position, when the labelling time is persued for a longer time. For this reason, we call this material FV-A gp55. It is important to note that the same basic results were reproduced with leukemic cells directly issued from the spleens of mice, proving that the observed situation is not only a consequence of the establishement of cells in culture. Incidently, we noticed that the yield of FV-A gp55 was higher in leukemic cells than in TF-A11 cells.

The presence of FV-A-gp55 in cells can reflect the expression of a set of nucleic acid sequences in the genome of the Friend anemia-inducing virus, which could be related to SFFV or entirely specific. This would imply that FV-P gp50-52 and FV-A gp55 may have the same fonction to play in the cell. SFFV sequences, which code for FV-P gp50-52 have been shown to contain at least 50 % of sequences of the MuLV helper (16). It might be conceivable that

the differences observed between FV-P gp50-52 and FV-A gp55 could be correlated to the differences in the "env" genes of each FV-A and FV-P helper viruses, as we have demonstrated in this work the existence of two distinct glycoproteins in their envelopes. To answer such questions, we are currently analyzing the oligopeptides resulting from proteolytic hydrolysis of the gp70-related glycoproteins.

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Note added in proof

While this manuscript was submitted, a work was published in Virology (1980), 102, 28-45). The results of this paper are in good agreement with those of our work and extend its significance.

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