Formation and characterization of FeLV ISCOMs

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Immunostimulating complexes (ISCOMs) have been prepared from feline leukaemia virus (FeLV) envelope proteins. The ISCOMs were characterized biochemically in SDS-poly-acrylamide gel electrophoresis showing the presence of proteins of estimated molecular weights of 15 000, 27 000 and 70 000. Immunoblotting showed that both the transmembrane protein p15E and the external glycoprotein gp70 (making up the gp85 protein) were present in the ISCOM. Furthermore, a degradation product of gp70 with an estimated molecular weight of 32 000 was identified in the immunoblot. The FeLV ISCOM was shown by electron microscopy to have the characteristic cage-like structure of an ISCOM with a mean diameter of 37 nm. About 10% of the total amount of gp70 in the culture fluid was recovered in the ISCOMs. The largest loss was encountered during the sedimentation of the virus. In a preliminary immunization experiment in mice the FeLV ISCOMs elicited after a booster gave a clear-cut immune response against gp70.

Introduction

Feline leukaemia virus (FeLV) belongs to the Retroviridae family, subfamily Oncovirinae. It has a worldwide distribution and may cause leukaemia in cats, but more often it causes an immunodeficiency syndrome. It is considered that the protective antigen is the glycosylated envelope protein with a molecular weight of 70 000 (gp70)[1]. Like most other retroviruses, the envelope of FeLV is first formed as a precursor protein (gp85). This is cleaved with a trypsin or a trypsin-like cellular enzyme of gp70 and p15E during intracellular transport to the plasma membrane before budding out with the virus particle. Gp70 being the peripheral part of the envelope protein, is to a great extent detached from the transmembrane protein p15E and the virus particle, and is found in the cell culture medium; p15E, however, remains integrated in the virus envelope.

Recently it was shown in an immunization experiment in cats by Osterhaus et al.[2,3] that gp70 integrated into an immunostimulating complex (ISCOM)[4] could induce protection to challenge infection in cats. The immunization experiment was extended to a larger experiment including 140 cats[3,5]. In this experiment about 85% of the ISCOM-immunized cats developed virus-neutralizing antibodies, available vaccine developed neutralizing antibodies. It had earlier been shown by Essex et al.[6] and later by others[7,8] that cats with neutralizing antibodies to FeLV do not develop disease, i.e. they have protective immunity to

FeLV. It has also been shown with a monoclonal antibody that gp70 contains at least one neutralizing epitope[1] and that this epitope is conserved in the three known serotypes.

In the present work we describe the preparation of ISCOMs containing FeLV gp70, and we characterize them as regards their morphology, their content of gp70, p15E and a breakdown product of gp70 migrating as a 32 000 molecular weight protein in an SDS-polyacrylamide gel electrophoresis. A preliminary immunization study in mice is also documented and the prospects of a vaccine are discussed.

Materials and methods

Materials

The detergent MEGA-10 (*n*-decanoyl-methyl glucamine)[9] was synthesized according to the method of Hildreth[10]. MEGA-10 was used as a 20% stock solution in water. Quil A 'Spikoside' was from Iscotec AB (Luleå, Sweden). Quil A was used as a 10% stock solution in water. The crosslinker DTNP (dithiobis-*m*-nitropyridine) was used as a 0.2% stock solution in DMSO[11].

Antisera. A mouse monoclonal antibody (mAb) against gp70[2] was used in the detection and purification of gp70. A hyperimmune antiserum against p15E from Raucher murine leukaemia virus was a generous gift from Dr Oswald Jarrett, Glasgow, Scotland.

Cells and virus. The cat T-lymphoma cell line F422[12] was used for the propagation of FeLV. Cells were grown in RPMI 1640 (The National Veterinary Institute, Sweden) supplemented with 10% fetal calf serum, 100 IU penicillin and 100 µg streptomycin ml⁻¹. Cultures up to 5 litres were grown in Roller bottles or in Spinner culture flasks. Larger volumes of cultures were run in a fermenter equipment from Chemoferm AB, Sweden. Harvested cell culture fluid was freed from cells and cell debris by low-speed

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centrifugation at 4000 rev min⁻¹ for 30 min in a Sorvall rotor GS3. The supernatant was collected and concentrated 10–15 times by ultrafiltration through a pellicon filter (Millipore PTMK 00005) with an exclusion limit of 300 000. From the concentrate the virus was sedimented at 20 000 rev min⁻¹ for 2 h at 10°C in a Kontron TFA 20 rotor. The virus pellet was suspended in 10 ml 0.5 M Tris, 0.15 M NaCl pH 7.5 (TN) and layered over a 30% sucrose cushion. The virus was centrifuged at 28 000 rev min⁻¹ for 2 h at 10°C in a Kontron TST 28.38 rotor. This step was avoided for some virus batches in an effort to minimize the loss of gp70, due to shearing forces during centrifugation. From the stock solution the crosslinker (DTNP) was added to a concentration of 0.02% and incubated for 30 min at room temperature to stabilize the binding of gp70 to the membrane anchoring part, p15E.

Methods

Labelling of virus glycoproteins. To dividing F422 cells were added 400 μCi glucosamine (TRK.398, Amersham), in 250 ml RPMI 1640 medium free from glucose and supplemented with 10% fetal calf serum, over 24 h.

Formation of ISCOM. This was essentially performed as described before[4]. To a virus sample of approximately 200 mg in 8 ml TN, 0.9 ml of the stock solution of MEGA was added to a final concentration of 2% and left for 2 h at room temperature. The solubilized virus was layered on top of 10% sucrose in TN in a volume of 2 ml containing MEGA at a concentration of 0.2%, with a layer of 30% sucrose in TN underneath. The solubilized virus was centrifuged at 30 000 rev per min for 2 h at 20°C in a TST.41.14 rotor. The top fraction consisting of the sample volume plus the 10% sucrose layer was collected and Quil A was added to a concentration of 0.1%. The mixture was extensively dialysed against TN.

SDS-polyacrylamide gel electrophoresis. This was done in a 12% gel according to Laemmli[13].

Western blots. These were done according to Towbin *et al.*[14] using nitrocellulose filter paper from Schleicher & Schüll, FRG.

Purification of gp70. FeLV gp70 was purified by an immunosorbent technique from culture fluid media or from infected cells. A monoclonal antibody recognizing a neutralizing epitope on gp70[2] was coupled to CNBr-activated sepharose (Pharmacia, Sweden) following the manufacturer's description. Gp70 was eluted with 0.1 M Tris pH 11.0. Fractions containing protein were adjusted to pH 8.0 with 1 M HCl.

Quantification of gp70. A quantitative dot-immunoblot using affinity-purified gp70 and a monoclonal anti-gp70 antibody were used to determine the amount of gp70 in virus and ISCOMs. In the dot-immunoblot analysis a nitrocellulose filter holder from Bio-Rad (USA) was used.

Fluorography. To analyse the glycoprotein content of a virus preparation, virus labelled with tritiated glucosamine was electrophoresed in SDS–PAGE gels and soaked in scintillator fluid (Amplify, Amersham, England). The gel was dried. The radioactivity pattern was analysed by fluorography using Kodak X-AR 5 film.

Protein determination. This was performed by the method of Bradford[15] using bovine serum albumin as standard.

ELISA. The ELISA[16] was performed in microtitre Immunoplates I (Nunc, Roskilde, Denmark). The plates were coated overnight at 4°C with either highly purified gp70 at a concentration of 1 μg ml⁻¹ or with FeLV ISCOMs at a concentration of 3 μg ml⁻¹ in 50 mM carbonate buffer pH 9.6. All washings were made with phosphate-buffered saline (150 mM, pH 7.5) containing 0.2% Tween-20 (PBS-Tween). The plates were blocked with 5% dried milk in PBS-Tween for 1 h at 37°C. Antisera were diluted in PBS-Tween and incubated for 1 h at 37°C. Horseradish peroxidase conjugated rabbit anti-mouse and swine anti-rabbit (Dakopatts, Copenhagen, Denmark) were diluted 1:1000 in PBS-Tween and incubated for 1 h at 37°C. The enzyme reactions were visualized with tetramethylbenzidine (0.19 mg ml⁻¹) (Sigma, USA) and H_2O_2 (0.006%) in 0.1 M acetate buffer pH 6.0 and absorbance was measured at 450 nm with a Titertek Multiscan spectrophotometer (Flow Laboratories, Irvine, Scotland).

Electron-microscopic analysis. Samples of ISCOMs were applied to hydrophilic, carbon-coated grids. For dialysis the specimens were placed upside down on micro-droplets of 0.1 M phosphate buffer, pH 7.0 before negative staining with ammonium molybdate, pH 7.0. The specimens were studied in a Philips 300 electron microscope at 60 kV accelerating voltage to improve the contrast effect. Photographs were taken in through focal series with minimal beam exposure.

Results

Formation and characterization of FeLV ISCOMs

FeLV originating from the cat T-lymphoma cell line F422 was concentrated and enriched by centrifugation. The virus material was solubilized with the detergent MEGA and the envelope proteins were integrated into ISCOMs by the dialysis method.

Recovery. The amount of the envelope protein gp70 was estimated at different steps of purification by a quantitative dot-immunoblot method using a monoclonal anti-gp70 antibody with highly purified gp70 as a standard. The purification steps include: (1) concentration by ultrafiltration where little loss occurred; (2) sedimentation of virus by two centrifugations where most of the gp70 was lost, approximately 70%; (3) the preparation of ISCOMs with a centrifugation and a dialysis procedure where an additional 20% of the gp70 was lost. The remainder is <10% of the amount of gp70 of the starting material. The detailed results are given in *Table 1*.

Table 1 Relative recovery of gp70 at different steps of virus purification and ISCOM preparation as measured by a quantitative dot-immunoblot method

Different steps in the purification of virus and formation of ISCOMs	Percentage recovery of gp70 from	
	Original material	Preceding step
Tissue culture fluid from FeLV-infected cells	100	100
Ultrafiltration	90–100	90–100
Pelleted virus	30	30–33
ISCOM	5–10	17–33

a   b c   d

gp70 →   ← gp70
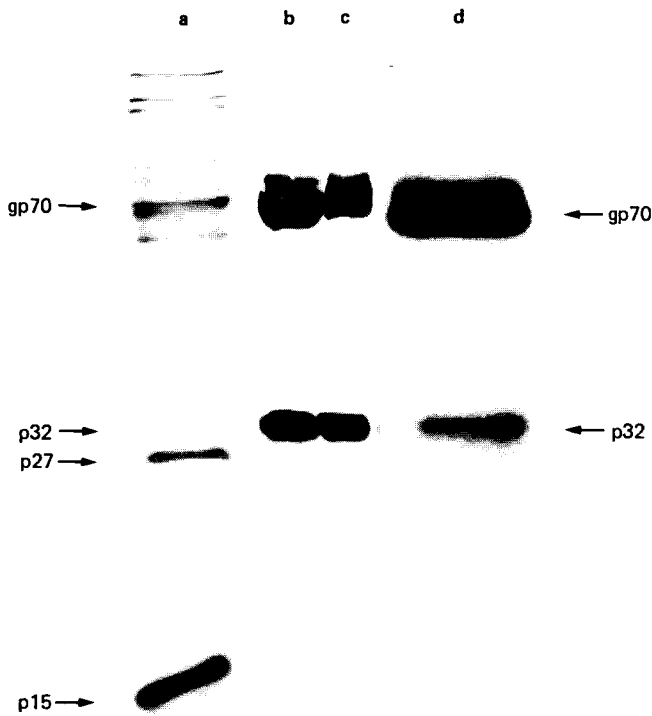
p32 →
p27 →   ← p32

p15 →

Figure 1 Analysis of FeLV and FeLV ISCOMs in SDS-polyacrylamide gel electrophoresis and in Western blot for the presence of the envelope protein gp70. (a) SDS-PAGE gel analysis of FeLV ISCOMs; (b) Western blot analysis of virus lysate using an anti-gp70 mAb; (c) Western blot analysis of FeLV ISCOMs using an anti-gp70 mAb; (d) autoradiogram of affinity-purified [³H]glucosamine-labelled gp70 separated in an SDS–PAGE gel

The protein pattern of the FeLV ISCOM. This was analysed in an SDS–PAGE and Western blot as described in Materials and Methods (*Figure 1*). Several protein bands are visible. The two most prominent bands in the ISCOM preparation migrated as proteins with molecular weights of 15 000 and 27 000 respectively. They probably represent the gag-coded proteins p15 and p27. Besides detecting a protein in the 70 kDa region, the monoclonal antibody to gp70 also identified a band in the 32 kDa region of the PAGE of the virus as well as of the ISCOM (*Figure 1b, c*). The 70 and 32 kDa bands were also identified by fluorography with immunosorbent-purified [³H]glucosamine-labelled gp70 separated in SDS–PAGE (*Figure 1d*).

By Western blot analysis the transmembrane protein p15E was detected in virus and ISCOMs using rabbit hyperimmune antiserum. Two bands were detected in the virus lysate, but in the ISCOM preparation only the 15 k band was visible (*Figure 2*).

ELISA analysis of FeLV ISCOMs. The FeLV ISCOMs were tested as antigen in ELISA against a monoclonal antibody to gp70 and against a rabbit anti-serum to p15E (*Figure 3*). The reading was plotted against the recriprocal antibody dilutions expressed as a natural logarithm. The behaviour and the slope of the curves for the two antisera are similar, indicating equal amounts of p15E and gp70 in the ISCOM preparation.

Electron-microscopic evaluation of FeLV ISCOMs. A relatively homogeneous population of ISCOM particles was observed by electron microscopy (*Figure 4*), demonstrating a characteristic cage-like structure. The diameter of the projections of ISCOM varied from 30 to 45 nm with a mean diameter of 37 nm. When ISCOM par-
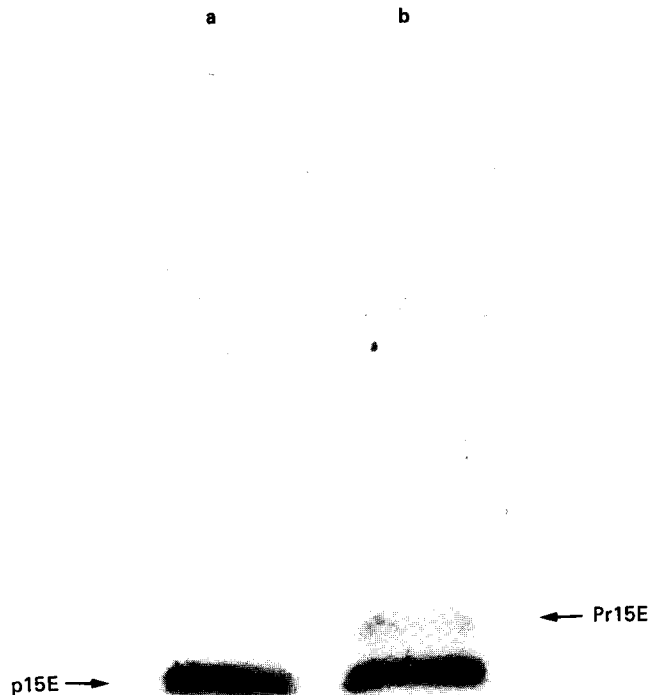
a   b

← Pr15E

p15E →

Figure 2 Western blot analysis of FeLV ISCOMs (a) and FeLV lysate (b) using a rabbit hyperimmune anti-p15E antiserum
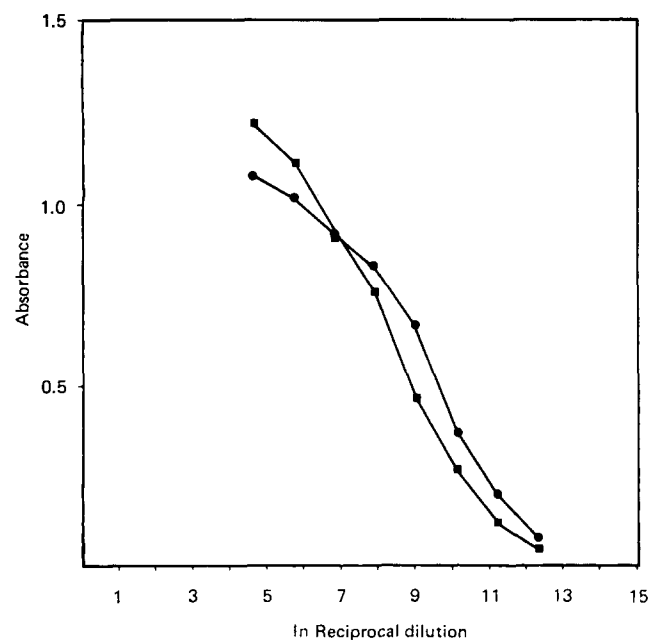

In Reciprocal dilution

Figure 3 Analysis of FeLV ISCOMs in ELISA for the presence of gp70 and p15E. The ELISA plates were coated with FeLV ISCOMs and incubated with different dilutions of the anti-gp70 mAb (●) and the anti-p15E antiserum (■) (see Materials and methods)
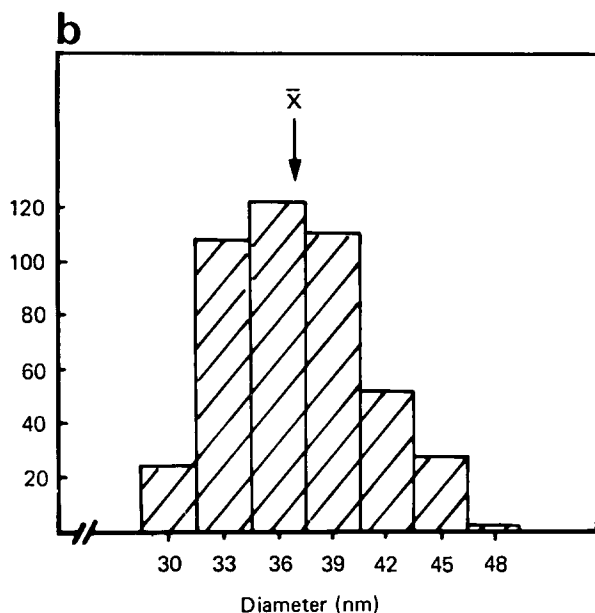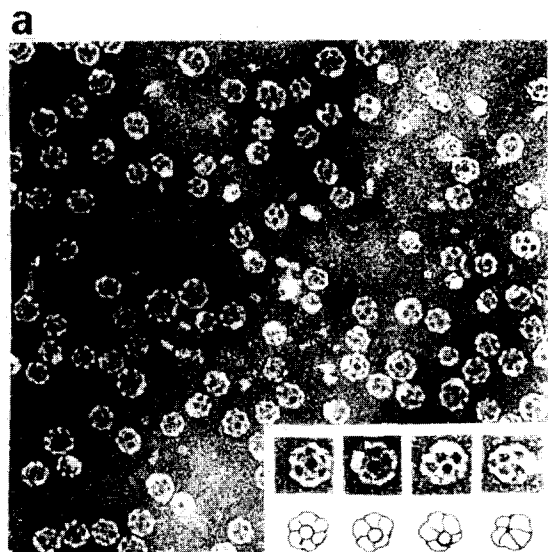
Discussion

Like most retroviruses, the peripheral and glycosylated part of the envelope protein gp70 of FeLV detaches from the anchoring and transmembrane part—p15E. This fact makes it difficult to isolate and to assemble gp70 to micelles, or to ISCOMs, by hydrophobic interactions to enhance its immunogenicity. The necessity of presenting immunogens in defined physical forms with multimeric presentation of the antigen has been reviewed[17]. The most immunogenic of such complexes seems to be the ISCOM[18]. However, an experimental ISCOM vaccine based on gp70 has already been shown to protect cats against challenge[2]. This vaccine was essentially prepared as described here, with a high amount of starting material and a low yield.

From *Table 1* it is evident that the major loss of gp70 antigen occurs during the centrifugation steps. The monomeric, highly water-soluble gp70, with a comparatively low sedimentation coefficient, is readily separated from the faster-sedimenting virus particle. The addition of DTNP, which is used with the intention of stabilizing disulphide linkage between p15E and gp70[11], did not prevent a major loss of remaining gp70. In the procedure used here, DTNP had to be added after the first centrifugation to avoid precipitation. It might be worth testing whether DTNP is more useful if the first centrifugation is replaced with another concentration and purification step. The final recovery of gp70 (in our procedure for making ISCOM) was ⩽10% and virtually all losses occurred during the virus-isolation procedures, as found with the preparation of ISCOMs with gp51 of bovine leukaemia virus[19]. Therefore, alternative methods for making immunogenic complexes based on hydrophobic interactions such as protein micelles or virosomes would not solve this
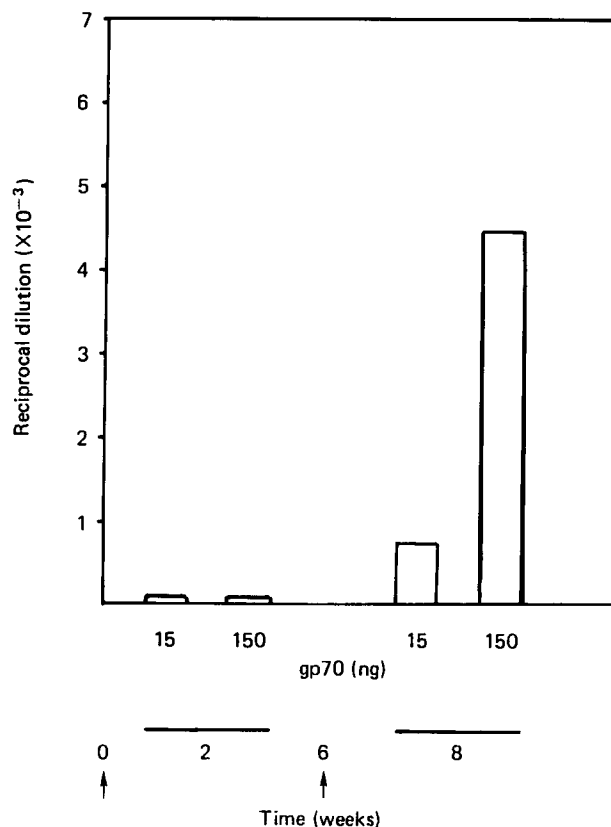
Figure 4 Electron microscopy of FeLV ISCOMs. (a) Electron micrograph of ISCOM particles, negatively stained by ammonium molybdate, showing projections of cage-like structures (× 125 000). A central, ring-like subunit of the shell of the ISCOM is shown in different projections in the insert, also demonstrated by the drawings. (b) Histogram of the distribution of the diameter of the projection of negatively stained ISCOM particles with an arithmetic mean value of 37 nm

ticles in different tilt angles were analysed they appeared slightly flattened.

Immunization of mice with FeLV ISCOMs. In a preliminary study two sets of five mice were immunized with FeLV ISCOMs with a total protein dose of 0.3 and 3.0 μg respectively. The gp70 content of FeLV ISCOMs in this study was estimated to be 5% of the total protein content corresponding to a dose of 15 and 150 ng of gp70 respectively.

The serum titres were tested against highly purified gp70 in an ELISA. Low but clear-cut serum titres were detected against gp70 by this assay. The end dilution titres for the two sets of mice after the first and second immunization were as shown in *Figure 5*.

Figure 5 Serum antibody titres to gp70 in mice immunized with FeLV ISCOMs as measured by ELISA. Arrows show first and second vaccination

problem. An efficient way to preclude described losses of gp70 is by use of an animal cell producing the complete envelope protein gp85, e.g. after transfection with an animal cell vector cloned with the *env* gene where the cleavage region of gp70 is deleted. The product of such a construct of the *env* gene for gp160 of HIV was efficiently integrated into ISCOMs.

The ISCOM preparation was analysed in PAGE and Western blot. In the PAGE the most prominent virus bands of virus proteins seem to be p15 and p27, while gp70 and p15E are less prominent. In the Western blot, on the other hand, gp70 and p15E respectively are readily recognized with a monoclonal antibody (gp70), which is recognizing a neutralizing epitope, and a polyclonal serum (p15E). This antibody (anti-gp70) is, therefore, potentially useful as a quality control tool for this experimental vaccine, as described by Merza *et al.*[19], who used neutralizing monoclonal antibodies to control an experimental BLV vaccine. The ISCOM preparation described here reacted also in ELISA with this antibody under a less denaturing condition than the Western blot. In the Western blot this antibody, as well as detecting a band migrating as a 70 kDa protein in the SDS–PAGE, also detected a 32 kDa band. This band was present both in the virus particle preparation and the ISCOM. It is probably a split or degradation product of gp70[20] and less is found in freshly prepared virus or ISCOMs than in older preparations. The other split product with an expected molecular weight of 30 000–40 000 has not yet been identified. FeLV ISCOMs comprising different proteins were analysed by electron microscopy by a size distribution of the ISCOM particles. Although somewhat flattened, as judged by different tilt projections in electron microscopy (*Figure 4a*), a variation in diameter (*Figure 4b*) may reflect a variation in volumes of the ISCOMs. The proteins in the shell of the cage-like ISCOM were efficiently exposed.

Another interesting observation is that the antibody recognizing p15E recognized two bands in the virus preparation but not in the ISCOM. The additional band has a slightly higher molecular weight in SDS–PAGE and may represent a precursor protein to p15E, as described for murine leukaemia virus[21]. The rabbit anti-Raucher p15E coprecipitates two proteins with estimated molecular weights of 12 kD and 15 kD. The presence of p15E was necessary for the coprecipitation with gp70. Virus preparations from F422 cells lacked p15E, but contained p12E, and failed to coprecipitate gp70[22]. This might explain the low yield of gp70 recovered from virus produced by F422 cells.

In a dilution experiment with the monoclonal antibody to gp70 and the antiserum to p15E, similar titration curves were obtained, which should indicate that the number of the two molecules were of the same magnitude. We would have expected the number of p15E molecules to exceed that of gp70 due to the great loss of gp70.

The FeLV ISCOMs produced as described here, readily induced antibody to highly purified gp70 in mice (*Figure 4*) after two doses containing as little as 15 ng gp70. The specificity of the antibody was confirmed in Western blot. It would, of course, be interesting to test in the natural host, i.e. the cat. Such experiments are presented in a subsequent paper[5].

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