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Lipopolysaccharide Induces Retroviral Antigen Expression in 129/J Mouse Lymphocytes: Evidence for Assembly of a Defective Viral Particle

JAN JONGSTRA AND CHRISTOPH MORONI*
Friedrich Miescher-Institut, CH-4002 Basel, Switzerland

In contrast to those of many other mouse strains, spleen cell cultures of 129/J mice do not release reverse transcriptase activity into the supernatant upon stimulation with bacterial lipopolysaccharide. We report here that lipopolysaccharide induced the expression of intracellular viral proteins in 129/J spleen cells. Furthermore, we found that stimulated spleen cells released retroviral particles. We conclude that 129/J mice are inducible with lipopolysaccharide but that the virus produced is a defective particle deficient in reverse transcriptase activity.

Certain B-lymphocyte mitogens, such as bacterial lipopolysaccharide (LPS) and lipoprotein, induce the appearance of endogenous xenotropic retrovirus in cultures of spleen cells from a variety of mouse strains (8, 18, 19). Induction by LPS requires the lipid A portion of the LPS molecule, which acts as the inducing determinant by combining with a specific receptor present on the cell membrane of a subset of B-lymphocytes (6, 17, 24). Accessory cells, such as T-lymphocytes or macrophages, are not required for induction.

Expression of xenotropic virus in mitogen-stimulated B-lymphocytes appears to occur only when accompanied by differentiation into immunoglobulin-secreting plasma cells. Mitogen-stimulated T-lymphocytes do not release infectious viral particles. These observations suggest that induction of endogenous retroviruses is not a simple consequence of de novo DNA synthesis but depends on the differentiated state of the lymphocyte and possibly on the process of B-cell differentiation itself (20).

In this study we examined the induction of endogenous viral genes by LPS in 129/J mice. Although the cellular DNA of these mice contains retrovirus-specific sequences and viral proteins that can be found in spleen extracts or serum, 129/J mice cannot be induced with LPS to release measurable amounts of reverse transcriptase activity (14, 25, 29). To explore this seemingly noninducible behavior of 129/J lymphocytes, we investigated whether LPS induced the appearance of intracellular viral proteins. Here we report that 129/J spleen cell cultures responded to LPS by expressing endogenous *gag* and *env* genes. Furthermore, we present data suggesting that stimulated 129/J lymphocytes are not defective in their ability to assemble viral particles but produce a particle defective in reverse transcriptase activity.

MATERIALS AND METHODS

Mice. BALB/c mice were obtained from the Ciba-Geigy animal breeding facility, Sisseln, Switzerland, and 129/J mice were obtained from Jackson Laboratories, Bar Harbor, Maine. They were used at 1 to 4 months of age.

Cell cultures. Spleen cell cultures were prepared with 2.5×10^6 viable nucleated cells per ml in RPMI 1640 medium containing 100 IU of penicillin per ml, 100 μg of streptomycin per ml, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and either no or 8% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y., batch no. K 763101 S). LPS (LPS-W from *Escherichia coli* O111:B4; Difco Laboratories, Detroit, Mich.) was added at 16 μg/ml when the culture was begun, and 5-bromodeoxyuridine (Calbiochem, La Jolla, Calif.) was added at 5 μg/ml after 17 h. For determination of the percentage of fluorescent cells and reverse transcriptase activity, five 1-ml cultures were pooled; for protein blotting experiments, cells were grown in flasks containing 25 to 30 ml.

Cells and viruses. NIH-3T3 cells and the mink lung fibroblast cell line CCL64 were obtained from N. Teich. NIH-3T3-AL cells are NIH-3T3 cells infected in this laboratory with ecotropic murine leukemia virus by cocultivation with LPS-stimulated AKR spleen cells. CCL64-BLS cells are CCL64 cells infected with xenotropic murine leukemia virus by cocultivation with LPS-stimulated BALB/c spleen cells. Friend leukemia virus (FLV) was purified by sucrose density gradient centrifugation from supernatants of Eveline cells, an infected STU mouse fibroblast line obtained from W. Schäfer.

Antisera. Anti-FLV antiserum was prepared against purified FLV in goats and was a gift from G. Hunsmann. This serum contains group- and type-specific antibodies (11). For immunofluorescence studies, this serum was used after in vivo adsorption: young BALB/c mice were injected intraperitoneally with 0.5

ml of anti-FLV diluted 1:3 in saline. Mice were bled on the next day from the orbital sinus, and serum was prepared by standard procedures. Rabbit anti-FLV gp70 (batch no. 9187) was a gift from G. Hunsmann, and rabbit anti-Moloney leukemia virus p30 (batch no. 3066) was provided by N. Müller-Lantzsch and Fan (21).

Immunofluorescence assay. Indirect immunofluorescence on acetone-fixed cells was carried out essentially as previously described (10). Cell suspensions were put on specially prepared slides, air dried, fixed in acetone for 10 min, and stored at −20°C. Several slides were prepared for each sample, which allowed us to restain parallel slides. Before immunofluorescence staining, the cells were rehydrated in saline for 15 min. Two wells were stained with antiserum diluted in saline containing 1% bovine serum albumin (fraction V; Fluka AG, Buchs, Switzerland). Normal serum or saline controls or both were included on each slide. Before counting, slides were coded for objective evaluation. Fluorescein isothiocyanate-conjugated anti-immunoglobulin G antibodies were purchased from Miles-Yedah, Israel, or Nordic, Holland.

Protein blotting. Protein blotting was done essentially as previously described (30), with minor modifications. Viral pellets were dissolved by boiling for 3 min in electrophoresis sample buffer containing 20 mM Tris, 1% sodium dodecyl sulfate, 2% β-mercaptoethanol, and 3% glycerol and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 15% polyacrylamide gels. After electrophoretic transfer of the separated proteins to filter paper (nitrocellulose; Millipore, France), additional protein-binding sites on the filter were saturated by incubation with 3% bovine serum albumin in saline for at least 1 h at 37°C. Filters were stained overnight at room temperature in a humidified chamber with rabbit antiserum or normal rabbit serum diluted 1:5,000 in saline containing 3% normal goat serum. After washing in two changes of saline for 30 min, filters were incubated for 2 to 3 h at room temperature with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G antibodies (Nordic) diluted 1:1,000 in saline containing 3% normal goat serum, followed by washing and the color reaction.

Separation of viable and nonviable cells. Cultured cells were pelleted in 15-ml glass Corex tubes at 1,200 rpm for 10 min at 4°C and suspended in 2 ml of 35% bovine serum albumin (Path-O-Cyte, Pentex; Miles Laboratories, Inc., Elkhart, Ind.). A total of 2 ml of RPMI 1640 medium was layered on top, and the gradient was spun in a Sorvall rotor, type HB-4, at 7,500 rpm for 25 min at 4°C. Viable cells were harvested from the interface, washed once in 50 ml of cold phosphate-buffered saline, and fixed on slides as described above for the immunofluorescence determination. Recovery of viable cells was routinely ~80%, with a viability level of >95%.

Enumeration of immunoglobulin-secreting cells. Cells secreting antibodies against sheep erythrocytes (SRBC) were determined by the Cunningham modification of the Jerne plaque assay (3). A counted number of cultured spleen cells were pelleted in a 15-ml Falcon conical plastic tube and suspended in 50 μl of RPMI 1640 medium containing 8% (vol/vol) SRBC (Graeub AG, Switzerland). After the addition of 50 μl of complement diluted 1:7.5 in RPMI 1640 medium, two 50-μl samples were added to microchambers sealed with a 1:1 mixture of paraffin and petrolatum and incubated at 37°C for 45 min. Clear plaques of lysed SRBC were counted visually. Guinea pig serum was used as the complement source and was absorbed twice with a 1/10 volume of packed SRBC before use.

Reverse transcriptase assay. Supernatants of LPS-stimulated spleen cell cultures were clarified by centrifugation for 10 min at 250 × *g* at 4°C and then for 10 min at 12,000 rpm in a Sorvall SS-34 rotor. The virus was pelleted for 45 min in a Beckman SW40 rotor at 40,000 rpm, suspended, layered onto a 15 to 60% linear sucrose gradient, and centrifuged for 2.5 h at 40,000 rpm in an SW40 rotor. Fractions with densities of 1.13 to 1.17 g/ml were pooled, the virus was pelleted and resuspended, and the reverse transcriptase activity was determined by assay conditions as described previously (19). The specific activity of the TTP in the reaction mixture was 28,500 cpm/pmol.

[^{3}H]thymidine incorporation. One-milliliter spleen cell cultures were incubated for 48 h and pulsed with 1 μCi of [^{3}H]thymidine (23 Ci/mmol; Amersham, Buckinghamshire, England) for the last 18 h. Then, 1 ml of 10% cold trichloroacetic acid was added, and the cultures were incubated for 30 min on ice. Samples (0.5 ml) were collected on a Whatman GF/C filter and extensively washed with 5% cold trichloroacetic acid, and radioactivity was determined by scintillation counting.

RESULTS

DNA synthesis and differentiation in 129/J spleen cultures. Virus induction by B-cell mitogens is correlated with induction of B-cell differentiation (20). We therefore stimulated 129/J and BALB/c spleen cells with LPS and assayed for induction of B-cell differentiation, DNA synthesis, and the release of reverse transcriptase activity in the supernatant. Since LPS activates B-lymphocytes polyclonally (1), the antibodies secreted from differentiated plasma cells are specific for many different antigens. For reasons of convenience, we chose to enumerate cells secreting antibody specific for SRBC to assess differentiation in our cultures. In contrast to BALB/c cells, 129/J cells failed to release reverse transcriptase activity upon stimulation with LPS, although the strains responded similarly with respect to DNA synthesis and B-cell differentiation (Table 1). We confirmed the previously published observation (25) that the addition of 5-bromodeoxyuridine to BALB/c cultures amplified virus release but had no effect on 129/J cells (data not shown). Thus, 129/J cells are competent to proliferate and differentiate in response to LPS but appear to be blocked with respect to virus production.

Induction of viral antigen in 129/J spleen cultures. It seemed possible that, despite the absence of reverse transcriptase activity in the

supernatant, LPS might still induce the production of intracellular viral proteins. We therefore measured by immunofluorescence, using in vivo-adsorbed anti-FLV antiserum, the appearance of viral antigen in LPS-stimulated 129/J cultures. After a lag phase of about 60 h, the percentage of antigen-positive cells increased rapidly in cultures containing LPS, and antigen-positive cells could be detected up to 155 h after culture initiation (Fig. 1). Cultures at later times were not examined. These stimulation experiments with 129/J cells were carried out in the absence of fetal calf serum since B-cell mitogenic substances in the serum also lead to induction of viral antigen (16).

To determine the specificity of the observed fluorescence, we tested replicate slides, using antiserum adsorbed with purified FLV or NIH-3T3-AL cells producing ecotropic AKR virus. Adsorption with FLV or infected NIH-3T3 cells reduced the number of fluorescent cells to background levels, whereas adsorption with uninfected NIH-3T3 cells had no appreciable effect (Table 2). These results indicate that the observed fluorescence was due to detection of viral antigen and was not the result of antibody binding to normal mouse cell components. As a further control, we stained replicate slides with a monospecific antiserum directed against purified FLV gp70 and found that LPS induced 13.5% gp70 positive cells. This is in good agreement with values found with the broadly reactive anti-FLV serum. Since the host range of mitogen-induced viruses in BALB/c and other mouse strains is xenotropic (22), we adsorbed the anti-

TABLE 1. *Virus induction, B-cell differentiation, and DNA synthesis in spleen cell cultures*

Strain	Addition to culture	Reverse transcriptase activity (cpm of [^{3}H]TMP incorporated)[a]	No. of SRBC-specific immunoglobulin-secreting cells per 10^6 viable cells[b]	[^{3}H]thymidine incorporation (stimulation index)[c]
BALB/c		ND[d]	11 ± 5	
	LPS	11,660 ± 1,853	49 ± 7	6.3
129/J		ND	28 ± 5	
	LPS	1,244 ± 171	98 ± 18	5.7

[a] Results are the average ± the standard deviation of two experiments performed in triplicate. The background value in the absence of added virus was 668 ± 482 cpm. Supernatants were harvested 96 h after culture initiation.

[b] Results are the average ± the standard deviation.

[c] Stimulation index is defined as the ratio of the counts per minute measured in LPS-stimulated cultures to the counts per minute in control cultures.

[d] ND, Not determined.

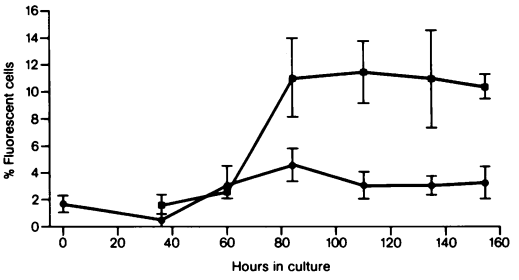

FIG. 1. *Serum-free spleen cell cultures from 129/J mice were prepared as described in the text and cultured for the indicated times, and the percentage of fluorescent cells was determined by using in vivo-adsorbed anti-FLV antiserum. Results are the average percentage of fluorescent cells ± the standard error from two experiments performed in duplicate. The value at 0 h was determined on freshly prepared spleen cell suspensions. Normal serum controls were 1.0 ± 0.4%. Symbols: ●, control cultures; ■, with LPS.*

serum with CCL-64-BLS cells. Virus-specific fluorescence could be expected to disappear after such an adsorption. Adsorption with infected mink cells reduced the percentage of fluorescent cells by more than 80%, whereas adsorption with uninfected mink cells had no effect (Table 2). These results show that, upon LPS stimulation, 129/J spleen cells express endogenous *env* gene products carrying group-specific antigenic determinants present on FLV and xenotropic viral gp70.

Protein blotting analysis of culture supernatant. We next examined whether the induced viral antigen became packaged into a particle and could be detected in a high-speed pellet. We chose to use a recently developed serological method for the detection of low amounts of proteins not containing a radioactive label (30). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteins are electrophoretically transferred to a nitrocellulose filter and stained with specific antiserum. This method is an attractive alternative to metabolic labeling followed by autoradiography since it is fast and obviates handling large quantities of radioactive material. When a high-speed pellet of 129/J culture supernatant was analyzed, staining with a monospecific rabbit anti-p30 antiserum revealed a clear band of 30,000 daltons (Fig. 2, track a). This band comigrated with viral p30 from purified xenotropic BALB/c virus (Fig. 2, track b). To characterize further the usefulness of the protein blotting procedure for the detection of viral p30, we analyzed 500 and 50 ng of total xenotropic viral protein in tracks b and c, respectively. No p30 band was visible in track c, indicating that a few hundred nanograms of virus was needed to detect the

TABLE 2. *Specificity of observed fluorescence on 129/J spleen cell cultures*[a]

Antiserum	Adsorbed with:	Fluorescent cells ± SE (%) in following culture:	
		Control	LPS stimulated
Anti-FLV	—[b]	2.1 ± 0.7	12.2 ± 1.7
	FLV[c]	<0.2	2.0 ± 1.3
	NIH-3T3[d]	2.1 ± 0.5	15.1 ± 3.2
	NIH-3T3-AL[d]	<0.2	0.6 ± 0.4
Anti-FLV gp70	—[b]	1.3 ± 0.4	13.5 ± 1.3
	CCL64[e]	1.2 ± 0.3	11.5 ± 0.9
	CCL64-BLS[e]	0.4 ± 0.1	2.6 ± 0.8

[a] Antiserum was adsorbed as indicated and used for fluorescence determinations on replicate slides made of cells after 110 h in culture and stored at −20°C. Results are the average of three duplicate experiments. Normal serum controls were 0.5 ± 0.2%. SE, Standard error.

[b] Mock adsorbed.

[c] Adsorption was for 1 h at 4°C at a final antiserum dilution of 1:10 with 120 μg of FLV per ml.

[d] Adsorption was for 1 h at 4°C at a final antiserum dilution of 1:10 with an equal volume of acetone-fixed NIH-3T3 cells or NIH-3T3-AL cells.

[e] Adsorption was for 1 h at 4°C at a final antiserum dilution of 1:50 with a 0.5 volume of acetone-fixed CCL64 cells or CCL64-BLS cells.

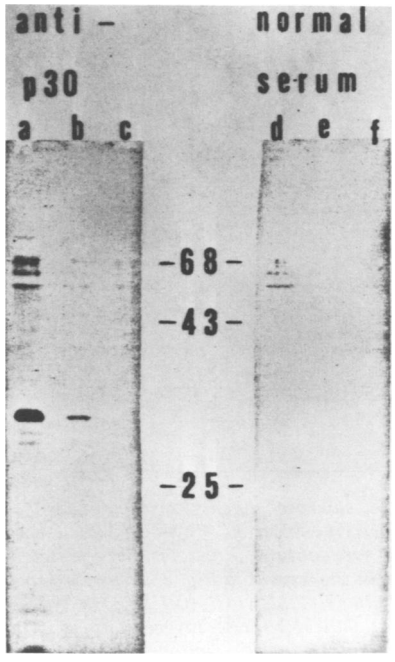

FIG. 2. *Protein blotting analysis of 129/J culture supernatant and purified xenotropic virus. Blots were stained with anti-p30 or with normal rabbit serum. Particles were pelleted as described in the text for the reverse transcriptase determination. In tracks a and d, pellets from 3 ml of LPS-stimulated spleen culture supernatant were analyzed. Tracks b and e received 500 ng of purified mouse xenotropic virus isolated from LPS-stimulated BALB/c spleen cultures and propagated in mink lung cells; tracks c and f received 50 ng. The middle column indicates the position of marker proteins bovine serum albumin, ovalbumin, and chymotrypsinogen, with their respective molecular weights given in thousands.*

presence of p30. Staining duplicate samples with nonimmune rabbit serum revealed only some nonspecific staining of high-molecular-weight bands present in the 129/J culture supernatant (Fig. 2, track d). Tracks e and f, containing 500 and 50 ng of purified xenotropic virus, respectively, did not show nonspecific bands.

To show that the p30 molecule present in pellets of culture supernatants was, in fact, part of a viral particle and not brought down by cell debris, we determined the density of the particle containing the antigenic material by sucrose gradient centrifugation. Pellets prepared from individual gradient fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and examined as described above. As shown in Fig. 3, tracks a through i, p30 could be detected in only two fractions, analyzed in tracks e and f and corresponding to densities of 1.15 and 1.13 g/ml, respectively. Track j contained 500 ng of purified xenotropic virus. Staining with normal rabbit serum revealed only some high-molecular-weight bands, as described in the legend to Fig. 2 (data not shown). Parallel analysis of LPS-induced virus from BALB/c cultures revealed the presence of p30 in particles banding at densities of 1.16 to 1.15 g/ml. Thus, stimulated 129/J cultures produced viral particles with slightly lower densities in comparison with the particles isolated from BALB/c mice.

Electron microscopy of LPS-stimulated spleen cells. Thin sections from LPS-stimulated spleen cells were examined by electron microscopy for the presence of virus particles. We observed rare budding particles with the morphology previously described for immature type C virus (Fig. 4). These observations show that retroviruses can assemble in and bud from stimulated 129/J spleen cells.

DISCUSSION

We have previously reported that 129/J spleen cell cultures do not release measurable amounts of reverse transcriptase activity into the supernatant upon stimulation with LPS, in contrast to cultures from a variety of other mouse strains (17, 20, 25). The defect responsible for this behavior might be due to the different genetic background of 129/J mice. Alternatively, differences in the endogenous proviral sequences in 129/J cellular DNA, as compared with those present in other, inducible strains, might be the

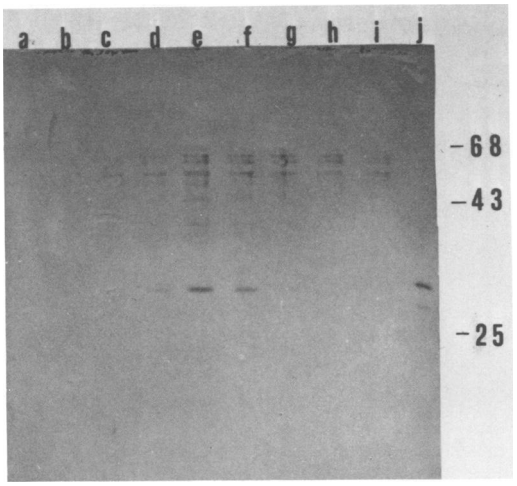

FIG. 3. *Sucrose density gradient analysis of a high-speed pellet from 40 ml of 129/J spleen cell culture supernatant. Particles were pelleted as described in the legend to Fig. 2, suspended in 100 μl of NET buffer (0.1 M NaCl, 0.01 M Tris-hydrochloride [pH 7.4], 0.01 M EDTA), layered onto a 10-ml linear gradient of 15 to 60% sucrose in NET buffer, and spun for 2.5 h at 40,000 rpm at 4°C in a Beckman SW40 rotor. Ten 1-ml fractions were collected by pumping from the bottom. After the density was determined, the fractions were diluted with 11 ml of cold NET buffer, and the material present was centrifuged for 30 min at 40,000 rpm and dissolved in 50 μl of 1% SDS at 37°C for 30 min. After the addition of 25 μl of 3× electrophoresis sample buffer, samples were layered on a 15% polyacrylamide gel and run at 110 V for 4 h. Tracks a through i were individual gradient fractions, with tracks a and i containing the bottom and top fractions, respectively. Track j contained 500 ng of purified xenotropic virus. Blots were stained with anti-p30. Numbers at the right indicate molecular weights of marker proteins as in Fig. 2.*

cause of this apparent noninducible behavior. Studies in other mouse strains have revealed that certain host functions are prerequisites for mitogen induction of virus expression. In BALB/c mice, mitogen-induced virus release takes place only when accompanied by differentiation of small B-lymphocytes into immunoglobulin M-secreting plasma cells (20). Male CBA/N mice, which have a recessive X-chromosome-linked genetic defect affecting B-cell differentiation, respond poorly to LPS both with respect to proliferation and differentiation and with respect to virus induction (22). 129/J spleen cell cultures have levels of DNA synthesis and differentiation comparable to those of the inducible BALB/c cultures (Table 1). This suggests that the noninducible character of 129/J mice is not due to a gross cellular defect resulting in an abnormal response to LPS.

No infectious virus has been isolated in 129/J mice (13, 22), although several authors have reported the partial expression of endogenous viral genes. Thymocytes of 129/J mice express G_{IX} cell surface antigen, which was shown to be part of a viral gp70 molecule (28, 31), whereas extracts of spleens of these mice contain both gp70 and p30 (15, 29). It was also reported that the bone marrow of 129/J mice contains ecotropic viral gp70 (15). It is thus clear that functional genes coding for these viral proteins are present in the cellular DNA of 129/J mice and that they can be expressed spontaneously in vivo. To determine whether at least some of these genes were inducible with LPS, we performed fluorescence studies (Fig. 1 and Table 2). The results show that 129/J DNA contains LPS-inducible endogenous viral genes.

Our gradient analysis shows that the induced p30 molecule can be assembled into a sedimenting particle. We consistently found the density (1.15 to 1.13 g/ml) of this particle to be slightly lower than the typical density of retroviruses of 1.16 g/ml (7). It is noteworthy that for the particle induced from BALB/c cultures, we found a density of 1.16 to 1.15 g/ml. It remains to be seen whether the lower density of the 129/J particle is a reflection of its defective nature. Further evidence for viral particle assembly in 129/J mice comes from electron microscopic analysis of LPS-stimulated spleen cells, in which we detected particles budding from the cell membrane. The observed particles had the morphology previously described for immature type C viruses (4, 9). The frequency of these particles was low and comparable to that in BALB/c mice (18), and no quantitative analysis has been performed.

The fact that 129/J particles are defective in reverse transcriptase activity is not a result of our enzyme assay conditions, for the following two reasons: first, a variety of mouse viruses, including mitogen-induced ecotropic and xenotropic viruses from several mouse strains, are readily detected by the assay as described above; second, cocultivation experiments with fresh spleen cells or LPS-induced 129/J spleen cells have not resulted in the isolation of a replicating virus (13, 22; unpublished data). Taken together, the data presented in this paper suggest that LPS induces the appearance of retroviral particles, defective in reverse transcriptase activity, in spleen cultures of 129/J mice. We do not know whether the 129/J particle contains a nonfunctional enzyme molecule or whether 129/J mice have a defect in synthesizing or packaging this enzyme.

Using DNA complementary to AKR ecotropic viral RNA, Lowy et al. (14) found two classes of

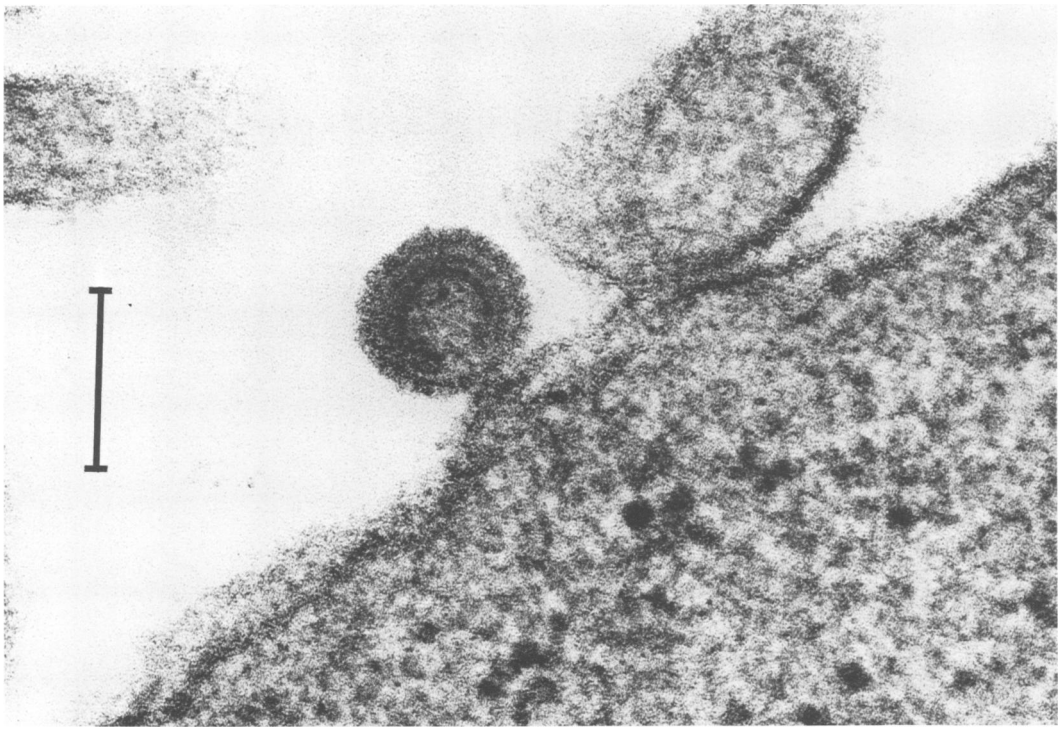

FIG. 4. *Cell pellets of LPS-stimulated 129/J spleen cell cultures were fixed with glutaraldehyde and postfixed with 1% osmium tetroxide. Ultrathin sections of dehydrated and embedded material were examined in a Phillips EM400 electron microscope. Bar = 0.1 μm.*

endogenous viral sequences in the DNA of high- and low-virus-yielding strains. 129/J DNA lacks the class representing ecotropic specific viral sequences but contains the 7 to 10 copies of sequences presumably representing xenotropic or as yet unidentified viruses or both present in all other strains. Until now, only a few of these proviral genes had been implicated in induced or spontaneous expression. Kozak and Rowe (12) found that iododeoxyuridine-induced expression of xenotropic virus from BALB/c and C57BL/10 mouse fibroblasts segregated with a gene closely linked to the *Dip-1* isoenzyme marker on chromosome 1. The spontaneous expression of xenotropic virus in NZB mouse spleen cells probably involves two genes (5, 13, 27). In AKR mice, it was shown that two genetic loci, *Akv-1* and *Akv-2*, control the spontaneous expression of ecotropic virus in vivo (23). These loci were shown to contain virus-specific sequences and thus presumably represent proviral copies (2, 26). Thus, some of the several endogenous viral copies are expressed and have been identified genetically. At present, we do not know whether the remaining copies represent viral genomes, the expression of which is more tightly controlled, partially defective copies, or genomes of viruses which have different biochemical and host range characteristics which would be difficult to detect by standard methods.

The retroviral 129/J particle described in this paper is possibly a representative of the group of defective endogenous viruses. The fact that it is inducible by LPS might facilitate direct study of this viral class. Further protein and nucleic acid studies are necessary to determine the extent of its defectiveness and its relation to the known mouse retroviruses.

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