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Properties of Density Gradient-Fractionated Peripheral Blood Leukocytes from Cattle Infected with Bovine Leukemia Virus

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Discontinuous bovine serum albumin gradients were used to fractionate peripheral blood leukocytes from bovine leukemia virus (BLV)-free and BLV-infected cows. The release of infectious BLV and spontaneous incorporation of [^{3}H]thymidine were not properties of density gradient-fractionated leukocytes from a BLV-free cow. When leukocytes from BLV-infected cattle were fractionated, B lymphocytes which spontaneously incorporated [^{3}H]thymidine could be separated as a distinct subpopulation from B lymphocytes which replicated infectious BLV. Density gradient fractionation of leukocytes from a cow with lymphosarcoma is also reported. A fall in lymphocyte count at the time of tumor development is attributed to the loss of B lymphocytes which spontaneously incorporate [^{3}H]thymidine.

In the accompanying paper (4) lymphocytes from cattle infected with bovine leukemia virus (BLV) were examined for the presence of surface immunoglobulin (S-Ig) and for their ability to form erythrocyte antibody complement (EAC) rosettes. Cells with these surface characteristics were found to contribute the major population of cells generated in BLV-associated persistent lymphocytosis (PL). Depletion of EAC rosette-forming cells (EAC-RFC) from peripheral blood leukocyte suspensions indicated that EAC-RFC were infected with BLV and spontaneously incorporated [^{3}H]thymidine after 3 days in culture. The purpose of the series of experiments reported here was to determine if BLV replication and spontaneous [^{3}H]thymidine incorporation are activities of one population of EAC-RFC or are functions of EAC-RFC subpopulations. In this report we describe subpopulations of cells obtained by density gradient fractionation of peripheral blood leukocytes from BLV-free and BLV-infected cattle.

MATERIALS AND METHODS

BLV-free and BLV-infected cattle with and without PL were selected from the defined groups of cattle previously described (4). The preparation of buffy coat leukocytes, the test for EAC-RFC, the syncytia infectivity assay, and lymphocyte culture techniques are described in the accompanying paper (4).

Discontinuous density gradients. Sterile buffered 35% bovine serum albumin (BSA), specific gravity 1.108, pH 6.9 (Path-o-cyte 5, Miles Laboratories, Kankakee, Ill.) was used to form discontinuous density gradients. Concentrations from 33 to 17% BSA in 2% increments were prepared by diluting the 35% stock solution with Eagle minimum essential medium. The gradients were formed in polystyrene tubes (16 by 125 mm) (Kimble Plastics, Toledo, Ohio) using a cushion of 1 ml of 35% BSA and carefully layering 0.8 ml of each dilution from 33 to 19% onto the surface. Buffy coat cells (5×10^8 to 7×10^8) in 1.5 ml of 17% BSA were layered on top of the gradients. The gradients were cooled in an ice bucket for 10 min before centrifugation at $500 \times g$ for 30 min at 5°C. After centrifugation the fractions were collected with a Pasteur pipette, including the cells at the lower interface with the fraction above. The fractions were then diluted with 5 ml of medium, centrifuged at $400 \times g$, resuspended in medium, and washed twice at $200 \times g$. After counting, the cell concentration was adjusted to 5×10^6/ml.

Immunofluorescent test for internal antigens of BLV. The immunofluorescent test used was previously described by Ferrer et al. (1). Briefly, leukocytes from density gradient fractions were pelleted and suspended in phosphate-buffered saline (0.01 M sodium phosphate-0.015 M NaCl, pH 7.2). Drops of the cell suspension were distributed on glass slides, air-dried, and fixed in acetone. The slides were incubated with either a BLV reference serum, a control serum from a BLV-free animal, or phosphate-buffered saline. After further incubation with fluorescein-conjugated goat anti-bovine 7*S* globulin and BSA-rhodamine counterstain, the slides were examined for the presence of fluorescent cells. At least 200 cells were counted to determine the percentage staining for internal antigens of BLV.

RESULTS

Table 1 shows the relevant peripheral blood cell characteristics of the animals used in this series of experiments. These cattle were selected for further study from the groups of animals described in our previous publication (4). BI-469 was BLV-free; BF-277 was infected with BLV but did not have PL, whereas BF-227 was infected with BLV and had PL. BF-187 also was infected with BLV, had PL up to and during the first study, but subsequently developed lymphosarcoma during the course of the present investigation.

BSA discontinuous density gradients were used to separate buffy coat leukocytes into 10 fractions. The cells recovered from each fraction were counted, and tested in the EAC rosette test, the syncytia induction assay, or placed in short-term culture to determine their level of spontaneous [^{3}H]thymidine incorporation after 3 days in culture. Figure 1 shows the density gradient profile of the BLV-infected cow without PL. Figure 1a shows the distribution of leukocytes on the gradient. As determined by examination of Wright-Giemsa-stained cell preparations, the least dense fractions (fractions 1 to 4) consisted almost entirely of mononuclear cells. Granulocytes accounted for 1% of the cells in fraction 5, increasing to 45% of the cells in fraction 6 and the majority of cells in succeeding fractions. The leukocytes from BF-277 were distributed on the gradient in two major peaks, the less dense consisting mainly of lymphocytes and the more dense consisting mainly of granulocytes. Figure 1b shows the distribution of EAC-RFC cells on the gradient. EAC-RFC had a peak of 64% in fraction 3, compared to 42% EAC-RFC in unfractionated peripheral blood lymphocytes prepared from the same sample of blood.

The distribution of syncytia-inducing cells on the gradient (Fig. 1c) shows that this activity also peaked in fraction 3 and is absent in fraction 5. This contrasts with the peak of spontaneous [^{3}H]thymidine incorporation (Fig. 1d) which occurred in fraction 5 and clearly indicates the presence of a population of cells which spontaneously incorporates [^{3}H]thymidine but does not replicate infectious BLV. Figure 2 is another gradient profile of the same animal which not only shows the distribution of cells inducing syncytia but also shows the distribu-

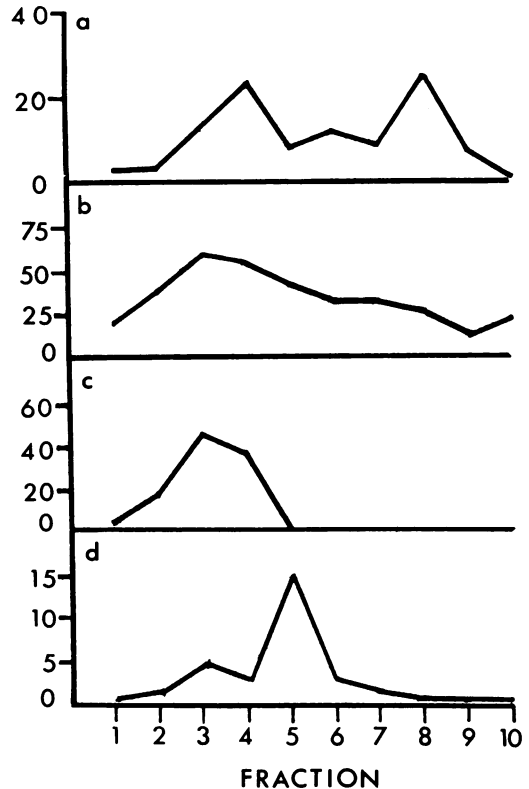

FIG. 1. *Discontinuous BSA density gradient fractionation of buffy coat leukocytes from BF-277 (BLV-infected without PL). Fraction 1 contains 17% BSA, and fractions are in 2% increments to 35% BSA (fraction 10). (a) Distribution of leukocytes on the gradient (expressed as percentage of total cells recovered); (b) percentage of EAC-RFC in each fraction; (c) distribution of syncytia-inducing activity (expressed as percentage of total syncytia formed); (d) distribution of spontaneous [^{3}H]thymidine incorporating activity (cpm × 10^3).*

TABLE 1. *Blood leukocyte characteristics of BLV-free and BLV-infected animals*

Animal	Total leukocytes/mm^3	Lymphocytes (%)	Granulocytes (%)	Monocytes (%)	EAC-RFC[a]
BI-469 (BLV-free)	5,500	81.0	16.0	3.0	23.0
BF-277 (BLV-infected, non-PL)	8,600	67.5	31.5	1.0	42.0
BF-227 (BLV-infected, PL)	36,700	94.5	4.5	1.0	67.0
BF-187 (BLV-infected, lymphosarcoma)	8,500	67.5	31.5	1.0	60.0

[a] EAC-RFC determined on sodium metrizoate/Ficoll-separated lymphocytes.

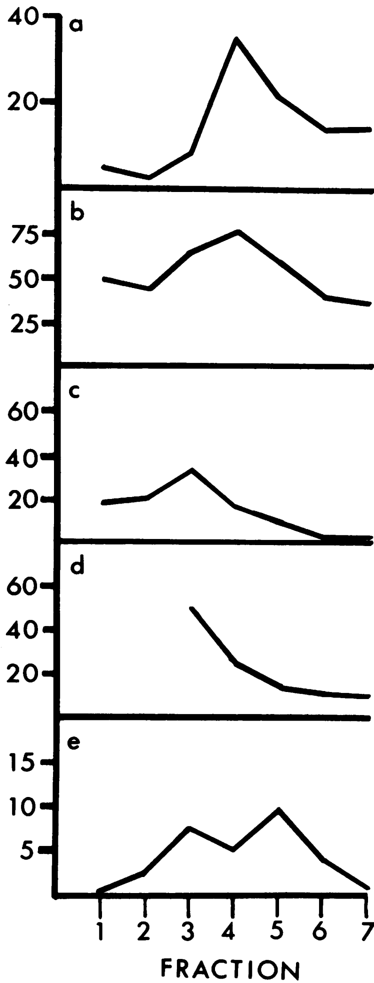

FIG. 2. *Discontinuous BSA density gradient fractionation of buffy coat leukocytes from BF-277 showing distribution of cells containing the major internal antigen of BLV. See Fig. 1 for explanations of panels a, b, and c. (d) Distribution of cells containing internal antigen of BLV (expressed as percentage); (e) distribution of spontaneous [³H]thymidine incorporating activity (cpm × 10³).*

tion of cells containing internal antigens of BLV (Fig. 2d). The distribution of antigen-containing cells in the fractions tested (fractions 3 to 7) was very similar to that of syncytia-inducing cells (Fig. 2c). The similarity between the gradient profiles in Fig. 1 and 2 indicates the reproducibility of the fractionation technique. In eight separate experiments in which buffy coat cells of BF-277 were separated on discontinuous BSA gradients, the peak of spontaneous [^{3}H]thymidine incorporation on day 3 occurred in fraction 5 six times, once in fraction 4, and once in fraction 6. The peak syncytia-inducing activity always occurred in fraction 2 or 3.

The density gradient profile of a BLV-infected animal with PL (BF-227) is shown in Fig. 3. As expected, a major peak of lymphocytes (fractions 2 to 5) was observed, with relatively few cells of the granulocyte series present in the more dense fractions. Both EAC-RFC and lymphocytes spontaneously incorporating [^{3}H]thymidine were distributed throughout the lymphocyte-containing fractions, whereas the cells releasing infectious BLV (Fig. 3c) had a more restricted distribution, with the peak again occurring in fraction 3. It is clear that, although fraction 5 has high activity in terms of [^{3}H]thymidine incorporation, it has comparatively few cells capable of inducing syncytia.

It was a consistent observation that syncytia induction by lymphocytes from BSA gradient fraction 3 is more efficient than by peripheral blood lymphocytes or buffy coat cells obtained from the same blood sample. In the experiment shown in Fig. 3, 358 syncytia were induced by 5 $\times 10^6$ cells from fraction 3, whereas only 2 were induced by the same number of buffy coat cells.

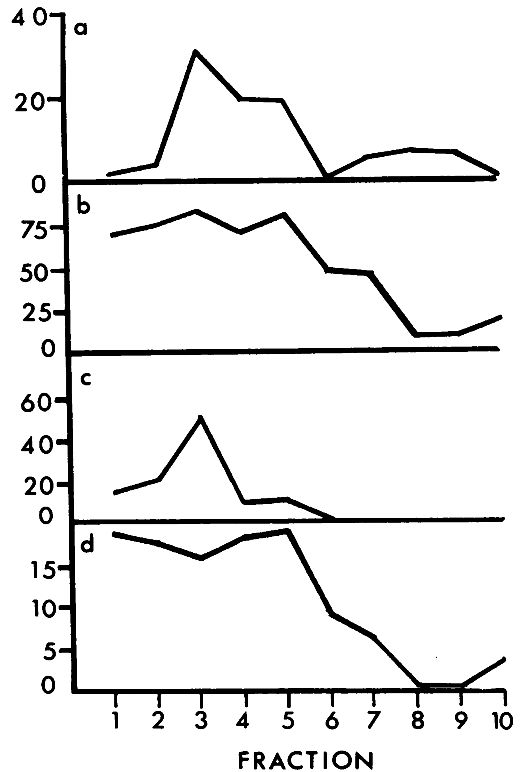

FIG. 3. *Discontinuous BSA density gradient fraction buffy coat leukocytes from BF-227 (BLV-infected with PL). Legend as in Fig. 1.*

This may be due to an inhibitory effect of cells which are separated from the syncytia-inducing population on the BSA density gradient. The addition of 2.5×10^6 cells from fractions 7, 8, or 9 to 2.5×10^6 cells from fraction 3 at the time of incubation with the bovine embryo spleen monolayer inhibited syncytia induction, whereas the addition of cells from fractions 1, 2, 4, or 5 did not.

Figure 4 shows that syncytia-inducing activity and spontaneous ^3H incorporation of thymidine were not properties of density gradient-fractionated leukocytes from an animal free of BLV infection.

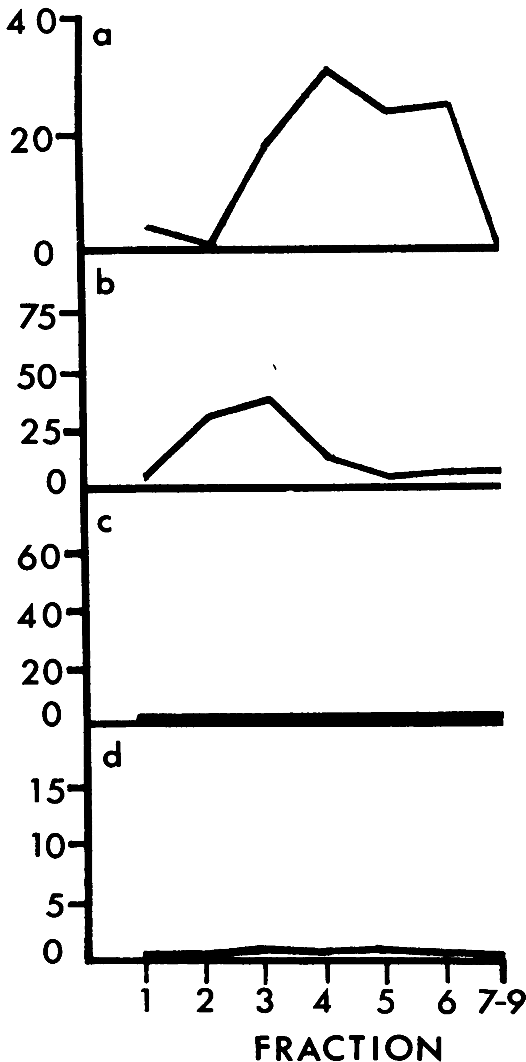

FIG. 4. *Discontinuous BSA density gradient fractionation of buffy coat leukocytes of BI-469 (BLV-free). Legend as in Fig. 1.*

Approximately 7 months after the studies reported in the accompanying paper (4) were completed, one of the animals (BF-187) in the BLV-infected PL group was noted to have enlargement of the palpable lymph nodes. A diagnosis of lymphosarcoma was made and confirmed by histological examination of sections of affected nodes. At that time the cow was 10 years old and had had PL for 8 years. The absolute lymphocyte counts for the preceding 2 years are shown in Fig. 5. A striking feature of this graph is the fall in absolute lymphocyte count which coincided with the appearance of clinical disease. This fall in the number of circulating lymphocytes in cattle with PL as clinical lymphosarcoma appears is not unusual (D. A. Abt, personal communication), although it does not appear to have been recorded in the literature. Three weeks after the tumors were noticed the cow had a total leukocyte count within the normal range and a normal differential leukocyte count. However the percentage of lymphocytes which were EAC-RFC remained elevated at 60% (Table 1). The BSA density gradient profile of its peripheral blood leukocytes at this time is shown in Fig. 6. There is a sharp peak of cells in fraction 3 which coincides with the peak EAC-RFC fraction. Syncytia-inducing activity peaks in fraction 3. However, no distinct peak of spontaneous [^{3}H]thymidine incorporation occurred, and the counts per minute did not exceed 2,500 in any fraction.

DISCUSSION

In the accompanying publication (4) we reported that the increase in peripheral blood lymphocyte counts in BLV-infected cattle was predominantly due to an increase in the number of cells with cell surface markers characteristic of B cells. The data also indicated that B cells from these animals were capable of replicating BLV and were responsible for the spontaneous uptake of high levels of thymidine. The results of the present investigation show that these are the activities of distinct subpopulations of B lymphocytes. Density gradient fractionation of peripheral blood leukocytes from BLV-infected cattle clearly shows that cells which spontaneously incorporate [^{3}H]thymidine may be separated from cells replicating infectious BLV.

It has been known for some time that buffy coat cells from BLV-infected animals replicate BLV upon short-term in vitro culture (5, 10); however, there was no further information as to the nature of the cells which were infected. Data from this and the accompanying paper show that not all PBL, nor even all B cells from

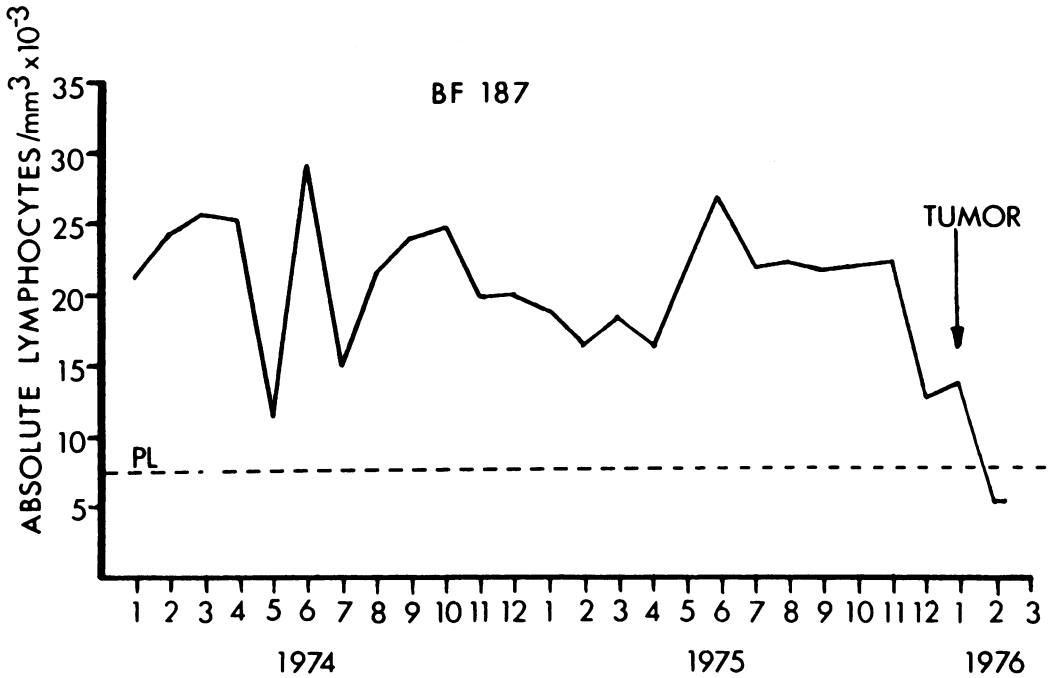

FIG. 5. *Monthly absolute lymphocyte counts for BF-187. Tumors were noted at the January 1976 bleeding. Dotted line indicates cut-off point for PL.*

infected cattle, are capable of releasing BLV, but this activity is restricted to a subpopulation of B cells which are found in the 19 to 23% BSA gradient fractions. These BLV-replicating cells have the same distribution on the gradient as cells capable of synthesizing an internal BLV antigen (Fig. 2). Restricted infection of T cells has been reported for murine leukemia viruses (2, 9) and vesicular stomatitis virus (6), and B-cell tropism has been reported for other lymphotrophic viruses such as Epstein-Barr virus (8) and cytomegalovirus (7).

It has been reported in the murine leukemia virus system that detection of the major internal antigens (gs antigens) is a reliable marker for the presence of the viral genome in the absence of complete virus (3). Our finding that the cells containing the major internal antigen of BLV make up only 15% of the cells in fraction 5 of the gradient from BF-277 suggests that the majority of B cells in this fraction are not infected with BLV but may be incorporating [^{3}H]thymidine in response to BLV or BLV-associated antigens to which they had been exposed in vivo. The fact that these cells are B cells from an animal making antibodies to BLV suggests that spontaneous [^{3}H]thymidine incorporation by this subpopulation of PBL may be indicative of in vitro expression of an antibody-producing function. Since the population of cells which spontaneously incorporates [^{3}H]thymidine appears to be larger in the animal with PL than in the BLV-infected animal without PL, it seems likely that PL is due predominantly to an expansion of this population.

The experiments reported here do not rule out the possibility that the cells synthesizing infectious virus may also undergo blastogenesis, since in the cow with PL (BF-227) spontaneous [^{3}H]thymidine incorporation was also a feature of fractions containing BLV-infected cells. Similarly, there was a small peak of [^{3}H]thymidine incorporation in fraction 3, the peak syncytia-inducing fraction, in both experiments with BF-227. However, it is clear that most of the spontaneous incorporation is attributable to cells not replicating BLV.

It has been a frequent observation that a fall in the absolute lymphocyte count of PL cattle coincides with the development of clinical lym-

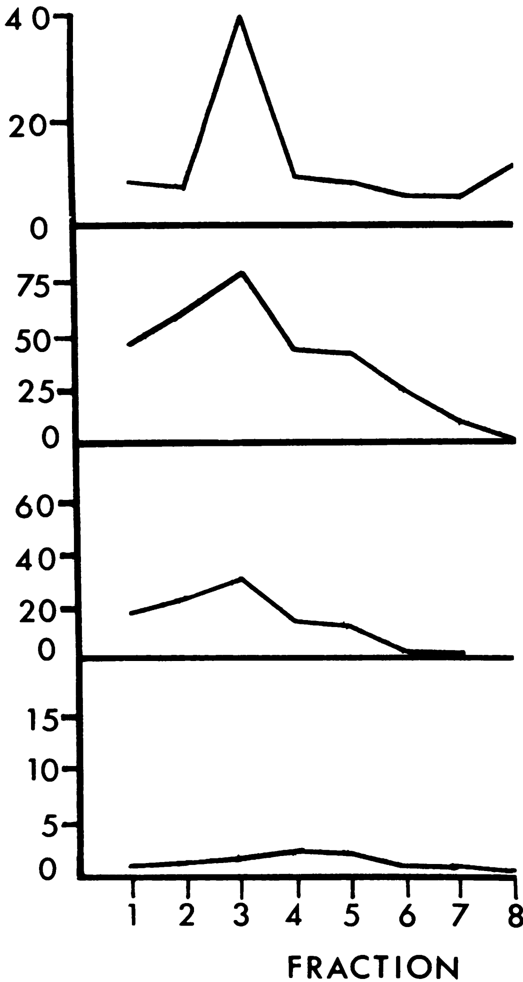

FIG. 6. *Discontinuous BSA density gradient fractionation of buffy coat leukocytes from BF-187 (BLV infected with lymphosarcoma). Legend as in Fig. 1.*

phosarcoma. This combination of events occurred in one cow included in this study. The BSA density gradient profile made after tumors had developed showed that the cow had an elevated absolute B-lymphocyte count, but a hemogram within the normal range. Little spontaneous [^{3}H]thymidine incorporation was evident, but the lymphocytes were capable of inducing large numbers of syncytia. Although a gradient profile was not made during the PL stage, it does seem likely that the fall in lymphocyte count was due to a loss of the spontaneously incorporating B cells.

More studies are clearly necessary to define the functions of the cells involved in BLV infection and to determine their relevance to tumor development.

ACKNOWLEDGMENTS

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