Moreover, since spherula cells are massively present in the brown bodies[11], it will be useful to study the role of the arylsulfatase in these coelomic cavity structures formed following antigenic stimuli.

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In vitro proliferation of human large granular lymphocytes with *v-raf/v-myc* recombinant retrovirus[1]

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Summary. The effect of infection with a retrovirus carrying *v-raf/v-myc* oncogenes (J2 virus) on the in vitro proliferation of human large granular lymphocytes (LGL) was investigated. LGL infected with J2 virus (J2LGL), unlike uninfected cells, grew with a proliferation peak eight days after infection. Such cells retained the morphology and functional properties typical of LGL. Furthermore, 5% of J2LGL produced virus the day after infection, whereas non-virus production was detectable five days later. These data indicate that J2 virus provides a transient mitogenic signal for LGL.
Key words. Large granular lymphocytes; retrovirus; infection; cell proliferation.

Natural killer (NK) cells have been described as a subpopulation of lymphoid cells with spontaneous cytotoxic activity versus a variety of target cells, including tumor cells, virus-infected cells and some normal cells[3]. Most of the NK activity has been recently associated with a subpopulation of $CD3^-$ lymphocytes, morphologically distinct from typical lymphocytes, which are known as large granular lymphocytes (LGL) because of their large size and the presence of characteristic azurophilic granules in their cytoplasm[4]. $CD3^-$ LGL, representing about 5% of mononuclear cells in the blood or spleen, express some cell surface markers characteristic of T cells, but also some markers associated with monocytes or granulocytes[4,5]. In addition, LGL can be grown in vitro in the presence of interleukin 2 (IL-2)[3,6]. Recently, numerous experimental evidence has shown that oncogenes, besides being involved in neoplastic transformation, also play a central role in cellular proliferation and/or differentiation[7–11]. For example, it has been reported that fresh tissues can be immortalized in vitro by appropriate recombinant retrovirus. Blasi et al.[12] found that in vitro infection of fresh murine bone marrow cells with the recombinant retrovirus J2 carrying *v-raf/v-myc* oncogenes results in selective immortalization of macrophage-like cells. Furthermore, it has been shown that infection of IL-2 or IL-3-dependent cell lines with a recombinant retrovirus, expressing the avian *v-myc* oncogene, abrogates the requirement for growth factors and suppresses *c-myc* expression[13].

In this report, we investigated the effects of infection with J2 virus on in vitro proliferation of human LGL.

Materials and methods. Isolation of LGL. LGL were obtained from buffy coats of blood donations of normal healthy volunteers, as previously described[4,6]. Briefly, mononuclear cells were separated by centrifugation on a Ficoll-Hypaque gradient (Pharmacia Fine Chemical, Uppsala, Sweden) and depleted of monocytes by adherence on a plastic surface. Nonadherent cells were applied onto a nylon-wool column, and eluted cells were then fractionated on a 7-step discontinuous gradient of Percoll (Pharmacia) at concentrations ranging from 40 to 60%[4]. LGL were collected from the low-density fractions (2 and 3 counting from the top). Contaminant T lymphocytes were further removed by rosetting technique with SRBC at 29 °C for 1 h[6]. Cell preparations contained an average of 85–90% LGL, as determined by morphological analysis of Giemsa-stained cytopreparations, and were $>60\%$ $OKM1^+$ and $<5\%$ $OKT3^+$ cells, as determined by flow cytometry.

Culture conditions. Culture medium was RPMI 1640 (Biofluids, Inc., Rockville, MD) supplemented with 10% human

AB serum (M. A. Bioproducts, Walkerville, MD). 0.1 mM nonessential amino acids (GIBCO, Grand Island, NY), 2 mM sodium piruvate (GIBCO), 2 mM glutamine (GIBCO), 50 µg/ml gentamicin (Sigma Chemical Co., St. Louis, MO), and 0,2 mM 2-mercaptoethanol buffered solution (Sigma). This medium will be referred to hereafter as complete medium. Human recombinant IL-2 was kindly provided by Dr S. Rudnick (Biogen, Cambridge, MA) and contained 8.5×10^5 U/mg of protein. LGL were suspended in complete medium containing IL-2 (500 U/ml) only during overnight infection. After infection the cells were cultured in 24-well plates (Costar Cambridge, MA) at a concentration of 1×10^6/ml. The LGL cultures were fed twice a week with complete medium.

Viruses. We used the recombinant J2 retrovirus which contains a complete *v-raf/v-mil* hybrid oncogene and a complete *v-myc* gene, consisting of the 5′ half of MH2 *v-myc* and the 3′ half MC29 *v-myc*[14, 15]. All infections were performed in the presence of a helper amphotropic virus 4070. Virus-containing supernatants (1 to 5×10^4 viral particles/ml), were diluted 1:2 with LGL suspension (final concentration 1×10^6 cells/ml). Polybrene (Sigma) (1:1000 final dilution) was added to the cell suspension to facilitate virus infection. After overnight incubation the cells were washed and cultured in fresh complete medium.

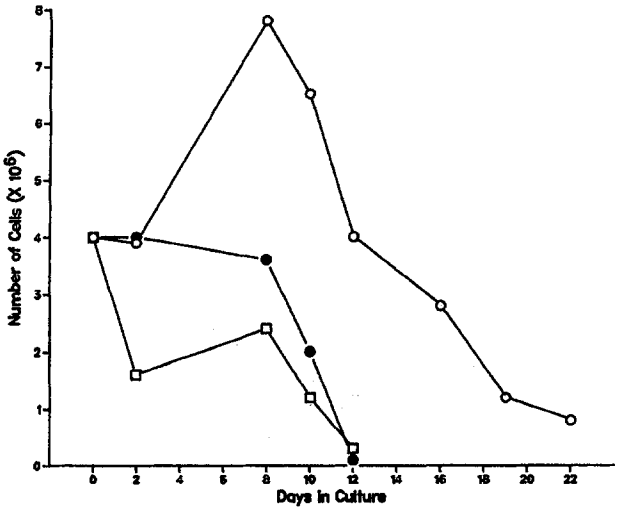
Assay for IFNs. The assay for IFN in supernatants from LGL was performed as previously described[16].

Flow cytometry analysis. We used the following monoclonal antibodies (MoAbs) to analyze the cell surface antigens of LGL: NKH1 (IgG1, Coulter Immunology); anti-Tac ($IgG2_2$) a gift from Dr T. Waldmann of NIH, Bethesda, MD; OKT3 (IgG2a, Orto Diagnostic Systems, Inc.); anti-Leu 11 (Becton Dickinson, Mountain View, CA). Immunofluorescence staining was performed as previously described[5]. Stained cells were analyzed with a cytofluorograf System 30-H (Ortho Diagnostic System, Inc., Westwood, MA) with a 2150 computer, modified to provide simultaneous measurement of forward and right angle light scatter (488 nm), red (>600 nm) and green (530 nm) fluorescence. Dead cells, identified by the uptake of propidium iodine (red fluorescence), were excluded from green fluorescence analysis. The percentage of positive cells in each fluorescence histogram was calculated by channel-by-channel subtraction of the appropriate control histogram.

Infectious centre assay (ICA). 10^5 3T3 fibroblast cells were plated in 60 mm dishes the day before the assay. Twenty-four h later, J2LGL were washed and treated with mitomycin (5 µg/ml) (Sigma) for 2 h in the dark. After inoculation the cells were washed and added to the fibroblast plates. Seven days later the number of transforming foci on 3T3 cell cultures was recorded.

Statistical analysis. Results were statistically evaluated using the Student's t-test. For clarity, tables and figures contain results of representative experiments, although all experiments were repeated, with similar results, at least eight times.

Results. Highly purified LGL from normal donors were resuspended in complete medium containing IL-2 (500 U/ml). After overnight infection with recombinant J2 retrovirus, carrying *v-raf* and *v-myc* oncogenes, the cells were washed and cultured in the absence of IL-2. Untreated LGL, as well as LGL infected with the helper virus 4070 alone (4070LGL), dies after approximately 12 days of culture. In contrast, in the cultures infected with the J2 virus (J2LGL) we observed a peak of growth at day 8, which was followed by a gradual decrease in cellularity (fig.). When day 8 J2LGL were analyzed by Giemsa staining, they showed a typical LGL morphology with the characteristic azurophil granules in the cytoplasm and expressed high levels of cytotoxicity against the NK susceptible K562 cells (data not shown). The phenotype of J2LGL was also analyzed by flow cytometry. These



Cellularity of LGL cultures at different days following infection. After viral infection, LGL (10^6/ml) were suspended in fresh complete medium without IL-2 and cultured in 24-well plates. The total number of viable cells was periodically monitored in J2LGL (○—○), 4070 LGL (□—□) or uninfected controll LGL (●—●). Data are the mean of triplicate samples and are representative of 8 experiments performed. The SEM was <10% of the mean (not shown).

Table 1. Phenotype of LGL following in vitro infection.

Cell	MoAb	Percent of positive cells Day 1	Day 5	Day 8	Day 12
Control LGL	Leu 11	59.1	54.8	76	–
	NKH 1	76.5	86.2	91.4	–
	OKT3	4.4	14	27	–
	Anti-Tac	NT	NT	93.6	–
J2LGL	Leu 11	52	46.8	70.8	79
	NKH 1	76.4	78.5	92	89.9
	OKT3	3.6	23.5	39.8	44.7
	Anti-Tac	NT	NT	69	44.8
4070LGL	Leu 11	49	46.4	78	–
	NKH1	74.5	79.5	83.9	–
	OKT3	3	11.6	14.9	–
	Anti-Tac	NT	NT	93.3	–

The percent of positive cells was determined by flow-cytometry 1, 5, 8 or 12 days after infection. Results of a representative experiment, of 8 performed. NT = not tested.

cells were highly stained by the NKH1 and Leu11 MoAbs, which recognize most common markers expressed on human LGL. With the time in culture, J2LGL became increasingly positive for OKT3, a MoAb specific for Pan T cells (table 1). Moreover, when compared with uninfected LGL, as well as with 4070LGL, we found a reduced percent of Tac positive cells expressed in the J2LGL population. This evidence suggested a possible down-regulation of the IL-2 receptor (rIL-2) by endogenous IL-2 present in the culture. Therefore, supernatants from J2LGL cultures were assessed for the presence of IL-2. Interestingly, significant amounts of IL-2 were found in these supernatants, whereas no Il-2 was detected in the control groups (data not shown).

Since previous studies have shown that LGL produce both IFN-γ and IFN-α in response to IL-2 stimulation[3, 4, 16], we next analyzed the supernatants for the presence of IFNs. As shown in table 2, the amounts of IFNs in 4070LGL cell cultures were comparable to those found in the controls, whereas significant enhancement in the levels of IFN-γ and IFN-α were detected in J2LGL supernatants on day 8. Two to four times differences in cell number per culture between

Table 2. IFN-production by infected and uninfected LGL

Cell	IFN (U/ml)					
	Day 2		Day 5		Day 8	
	IFN-γ	IFN-α	IFN-γ	IFN-α	IFN-γ	IFN-α
Control LGL	1000	700	1625	1250	20	12
J2LGL	520	400	2600	2000	1000	560
4070LGL	520	400	1000	700	100	65

Supernatants from cell cultures were harvested at various days after infection and tested for the presence of IFN-γ or α using a biological assay[16]. The results, expressed as International Antiviral Units of IFN per milliliter of cell supernatant, are the mean values of triplicate samples. The SEM was <10% of the mean (not shown).

Table 3. Number of virus-producing LGL determined by ICA

Cell	Number of infectious centers		
	Day 1	Day 5	Day 10
Control LGL	0	0	0
J2LGL	490[a] (4.9)[b]	20 (0.2)	0
4070LGL	0	0	0

The infectious centers of mitomycin-treated LGL were determined by ICA on 3T3 fibroblast cells. [a] The values represent the number of infectious centers in 10^4 cells tested. [b] The numbers in parentheses indicate the percent of virus-producing cells.

J2LGL and 4070LGL or control LGL (fig.) could not account for the difference in levels of IFNs produced (10 to 50-fold increase). Therefore, these data suggested that increased IFN production was not a result of the viral infection per se, but it was rather dependent on J2 virus expression. Following the initial burst of proliferation, J2LGL died after three weeks of culture. One possible explanation for this phenomenon is that J2LGL were progressively eliminated by cytotoxic and/or inhibitory mechanisms(s). Therefore, using an ICA we analyzed the proportion of LGL producing J2 virus at different times following infection. As shown in table 3, approximately 5% of J2LGL were able to produce virus one day after infection; however, no virus producing cells were detected at day 10. This finding indicated that, following initial infection, J2LGL lost the ability to produce infectious virus in culture. Since, we have consistently found that J2LGL produced significant amounts of IFNs, it was possible that the IFN produced in the cultures inhibited virus replication. Therefore, either lack of virus production or antiproliferative effects by IFN could account for the decreased proliferation of J2LGL, observed 2 to 3 weeks following infection.

Discussion. The role of c-oncogenes in cellular proliferation has been widely demonstrated in many experimental models[9,17]. Previous reports have shown, for example, that murine myeloid precursors cultured in the presence of exogenous growth factors (GF) responded with augmented self-renewal upon infection with recombinant retrovirus carrying *v-myc* or *v-src* oncogenes, suggesting a synergism between some viral oncogenes (*v-onc*) and certain GF. Recently, it has been shown that the combination of the two oncogenes *v-raf* and *v-myc* in J2 virus induced the selective proliferation of monocytic cells from fresh murine BM in the absence of a specific GF supplement[12]. Both *v-myc* and *v-raf* genes had to be expressed to induce cell proliferation, since viruses carrying only one oncogene did not promote cell growth. Therefore, it was proposed that *v-onc* expression might substitute the need for specific exogenous GF.

On the basis of such experimental evidence, in this study we investigated the effects of the retrovirus J2 on human LGL. The results showed that infection of human LGL with J2 virus induced a transient proliferation of these cells which were capable of growing in absence of GF. After the initial peak in proliferation, however, J2LGL progressively died in culture. When assessed for J2 virus production by ICA, only a small proportion of LGL was able to produce virus the day after infection, whereas no virus was any longer detectable the following days. This could be explained by the inhibitory effects of the endogenously produced IFN on J2 virus replication. Alternatively, the block in cell proliferation could be attributed to the killing of virus-infected cells by uninfected LGL. This hypothesis is in agreement with previously published data, demonstrating that a variety of normal cells become targets for LGL upon infection with viruses[3]. The results of the assay using J2 virus transformed 3T3 fibroblasts as target for normal LGL support this conclusion. Both normal and IL-2 activated LGL showed in fact higher levels of cytotoxicity against J2 infected 3T3 fibroblasts rather than non-infected fibroblasts (data not shown). On the other hand, the release of lymphokines during the lytic process could, in turn, represent the stimulus responsible for the observed proliferation. When J2LGL supernatants were analyzed for the presence of IL-2, we found appreciable amounts of such GF, thus suggesting a possible role of IL-2 on the cell growth observed. We are currently attempting to clone the LGL population after infection, to assess the relevance of the cytotoxic mechanisms in the elimination of J2 infected LGL.

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