Divergence between Immunosuppression and Immunocompetence during Virus-Induced Leukemogenesis

(mice/Friend leukemia virus/*Vibrio cholerae*/sheep erythrocytes)

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ABSTRACT Mice were infected with Friend leukemia virus and later immunized with either *Vibrio cholerae* vaccine or sheep erythrocytes. The primary antibody response to the bacteria (as judged by the number of plaque-forming cells) was slightly enhanced by the viral infection, whereas the response to sheep erythrocytes was inhibited. The difference appeared due to sensitization of mice to antigens crossreacting with those of sheep erythrocytes; no natural immunity to *V. cholerae* is detectable. However, the response of mice infected with Friend leukemia virus to a secondary challenge with the cholera bacteria was markedly inhibited. Even though the number of plaque-forming cells during the primary response was not reduced, accumulation of the cells in distinct splenic foci was suppressed. These results suggest that the effect of Friend leukemia virus on immunocompetent cells is selective. The immune response appears to be susceptible to leukemia virus-induced immunosuppression only when there has been a previous stimulation of immunocytes by antigen.

Experimental infection of susceptible mice with RNA murine leukemia viruses resulting in malignant proliferative disease of the reticuloendothelial system is accompanied by a non-specific inhibition of the immune response to a large number of antigens. Ample evidence has now accumulated in several laboratories concerning such immunosuppression and has recently been reviewed by Notkins *et al.* (1). It has been suggested that leukemia virus-induced immunosuppression may be one of the important factors in tumorigenesis, i.e., the generalized impairment of immunity includes the potential immune defense of the individual against the virus itself, virus-induced tumor antigen, or both, thus paving the way for the neoplastic process. However, as shown by the present study, the immunosuppressive effect of murine leukemia viruses may not be as unequivocal as previously believed.

The two antigen models we chose for the comparative studies on FLV-induced immunosuppression, namely, the gram-negative bacterium *Vibrio cholerae* and sheep erythrocytes, differ in one major aspect. There is a variable but substantial number of "background" plaque-forming cells (PFC) in the spleen of nonimmunized mice that form antibodies to erythrocytes, resulting probably from continuous sensitization of mice with crossreacting bacterial antigens (2, 3). A nonspecific stimulation of cell differentiation results in an

increase of such "background" PFC, even without inoculation with sheep erythrocytes (4, 5). On the other hand, there is no detectable "background" to *V. cholerae* in mice; even vigorous nonspecific stimulation with known stimulators fails to elicit any detectable antibody-forming cells (6, and unpublished data). In contrast to the situation with sheep erythrocytes, the reaction of "virgin" antigen-reactive cells to the first immunization with the cholera bacteria can be considered a true primary response.

Immunity to sheep erythrocytes, as measured by the hemolytic PFC technique, has been widely used in previous studies of the mechanism of immunosuppression during viral leukemogenesis. Infection of mice with FLV before immunization suppressed 90% or more of the subsequent response to sheep erythrocytes in the spleen of mice (7, 8). Other leukemia viruses had similar effects (1).

Previous experiments from this laboratory demonstrated that splenic antibody-forming cells differentiate as isolated foci (9-12). One focus may contain 50-2000 PFC (11, 12). Indirect evidence suggests that each focus is created by an immunologically specific antigen-reactive cell. Thus, comparison between relative suppression of focus and PFC number, respectively, by FLV could reveal whether the virus affects immunocompetent cells directly or through their progenitors.

MATERIALS AND METHODS

Animals and infection

BALB/c mice of both sexes, 4-6 weeks old, were used throughout these experiments. The source, maintenance, and passages of FLV and the determination of virus concentration calculation of the median infectivity dose (ID_{50}) were described previously (7). Infected control mice were immunized intravenously either with 5.0 μ g of heat-killed *V. cholerae* vaccine or with 10^8 sheep erythrocytes.

Plaque assay for antibody-forming cells

We counted the cells that form antibodies to sheep erythrocytes in suspensions of individual spleen cells, using the agar plaque technique exactly as described by Jerne *et al.* (2). For assay of antibody-forming cells to *V. cholerae*, the immunoplaque technique was modified as follows. Spleen cells to be assayed are mixed with viable bacteria in melted agar and poured into Petri dishes containing a base layer of nutrient agar. The dishes were incubated and treated with complement at 37°C.

Abbreviations: FLV, Friend leukemia virus; PFC, plaque-forming cells.

Cells releasing bacteriolytic antibody are detected as plaques in a homogeneous layer of growing bacteria. The typical immune responses detected by the plaque assay methods in the spleen of immune mice are illustrated in Fig. 1. The initial increase of antibacterial and anti-sheep erythrocyte PFC between the second and fourth day after immunization is similar. However, the end of the log phase of the former is followed by a plateau and another slow increase, so that the peak number of antibacterial PFC is not attained until the tenth day.

Assay for splenic foci and areas

The method for detection of antibody foci (12) in sections of frozen spleen was adopted from previous studies with other bacteria (10). The spleens were frozen rapidly and placed in a cryostat at -18°C . Thin longitudinal sections ($6-7\ \mu\text{m}$ thick) were cut and transferred rapidly—inside the cryostat—onto test agar plates containing viable bacteria. Incubation of the plates at 37°C , followed by treatment with complement, results in focal bacteriolysis under the splenic sections. The sharp foci contrast with the surrounding homogeneous bacterial growth, developed in a few distinct areas of the spleen (Fig. 2). Comparison of focus distribution in serial sections through the organ permits determination of the number of foci per individual splenic area.

RESULTS

Infection of mice with FLV 4-days before immunization resulted in about 80% suppression in the number of anti-sheep erythrocyte PFC, as compared with uninfected control mice (Table 1, Exp. I). On the other hand, the 4-day response to *V. cholerae* was not inhibited under the same experimental conditions. The 10-day or peak immune response to the bacteria was even enhanced, as compared to the controls (Table 1, Expt. III). Similar results were obtained when the antigens were injected together into each mouse and PFC of both specificities were counted in aliquots of the same spleen (Table 1, Expt. II). Although an individual infected spleen might show only 20% of the control immune capacity to sheep erythrocytes as controls, the response of the same spleen cells to the bacterial antigen was higher than infected controls.

A 10-fold increase in the dose of FLV, or prolongation of the

FIG. 1. Typical response of plaque-forming cells in the spleen of adult mice after a single immunization with sheep erythrocytes (O) or *V. cholerae* (●), respectively. Spleens from 5–10 mice were assayed per point. Note the number of PFC to sheep erythrocytes, and none to *V. cholerae* in control mice.

time interval between infection and immunization, had no appreciable effect on the immune response to *V. cholerae* (Table 2). Even the pathologically changed and enlarged spleen at 12 days after infection (average spleen weight of 1400 mg, as compared to an average of 154 mg in uninfected BALB/c mice) contained normal numbers of reactive cells to the bacteria.

Experimental evidence indicates that antigen-reactive cells to sheep erythrocytes are previously sensitized by natural stimuli, whereas the anticholera antigen-reactive cells are not (see *Introduction*). Thus, the selective effect of FLV on the immune response to these two immunogens could be tentatively explained by an assumption that "virgin" cells that have not been exposed to an antigen are not susceptible to virus infection. If this presumption is correct, the secondary response to *V. cholerae* should be inhibited by FLV. To test

FIG. 2. Longitudinal section from the spleen of a mouse immunized with *V. cholerae*. The section was tested for bacteriolytic activity as described in *Methods*. Zones of bacteriolytic antibody production appear as sharp foci assembled in three distinct areas of the spleen. Assay of serial sections from the same organ reveals the average number of foci in each area (numbers for this section are 8, 3, and more than 10 foci per area, respectively).

TABLE 1. Plaque-forming cell response, in FLV-infected mice, to sheep erythrocytes and *V. cholerae*

Expt.	Antigen	FLV infection*	PFC/spleen		
			Fourth-day assay		Tenth-day assay (anti- <i>Vibrio</i>)
			anti-SE†	anti- <i>Vibrio</i> ‡	
I	SE	—	108,000§ (83,000–167,000)	—¶	—
	SE	+	21,500 (10,000–39,000)	—	—
	<i>Vibrio</i>	—	—	252 (16–1,000)	—
	<i>Vibrio</i>	+	—	380 (24–400)	—
II	SE + <i>Vibrio</i>	—	100,000 (50,000–160,000)	5,220 (32–19,800)	—
	SE + <i>Vibrio</i>	+	21,400 (11,300–29,800)	6,570 (20–27,500)	—
III	<i>Vibrio</i>	—	—	—	13,000 (9,450–21,300)
	<i>Vibrio</i>	+	—	—	52,390 (25,000–95,700)

* Mice infected with 1–5 ID₅₀ of FLV, 3 days before immunization with sheep erythrocytes (SE), *Vibrio* (*V. cholerae*), or both.

† Sheep erythrocytes.

‡ *Vibrio cholerae*.

§ Average number of PFC in spleens of six mice, with range of individual values.

¶ Not done.

TABLE 2. Decrease in PFC response to *V. cholerae* in mice previously exposed for longer intervals or to smaller doses of FLV

	Control	Dose of FLV*		Interval between exposure to FLV† and <i>V. cholerae</i> (days)			
		10 ⁻¹	10 ⁻²	0‡	3	7	12
PFC/spleen (4 days after immunization)	385§ (63–1000)	540 (442–1600)	313 (52–650)	500 (263–758)	493 (88–741)	455 (84–888)	235 (59–417)

* Concentrations are expressed as serial dilution of spleen homogenate (20% w/v) from a stock of mice infected with FLV passage. Mice were infected with virus 4 days before immunization with *V. cholerae*.

† All mice were infected with 10⁻² dilution of FLV spleen homogenate.

‡ FLV and *V. cholerae* were injected on the same day.

§ Average from three to six mice, with range of individual values.

this possibility, mice were immunized with 1 µg of cholera vaccine. 8 weeks later, some of the mice were infected with 1–5 ID₅₀ of FLV and challenged 4 days later with a second

TABLE 3. Enhanced primary and decreased secondary PFC response to *V. cholerae* of mice previously infected with FLV

Mouse group*	FLV infection†	PFC/spleen	% of controls
Primary response	—	45,500 (13,100–115,000)	—
	+	115,000 (8,220–252,000)‡	252
Secondary response	—	289,000 (101,000–413,000)	—
	+	30,000 (3,780–81,000)	10

* Secondary response of mice to 5 µg of vaccine injected 8 weeks after primary immunization.

† Mice were infected with 1–5 ID₅₀ FLV, 4 days before immunization; control mice were not infected (—).

‡ Average number of PFC per spleen, 10 days after immunization (range of values for groups of six mice).

injection of vaccine, as were other primed uninfected control mice. Normal mice of the same age (both FLV-infected and uninfected) served as controls of the primary response. As is evident in Table 3, the secondary response was suppressed 90% by previous infection with FLV, whereas the primary response was enhanced.

The possible effect of FLV on differentiation of antibody-forming cells in isolated splenic foci was studied in parallel groups of mice immunized with *V. cholerae*, either FLV-infected or not. PFC were counted in spleens of one group and the number of foci was determined in splenic sections from another group (Table 4). The number of detectable splenic foci was about 60% lower than in controls, even though the PFC number in the spleens of the parallel group of mice was unaffected by FLV infection. The number of splenic areas in which the antibody foci and PFC accumulated was also the same in both infected and control spleens.

DISCUSSION

It seems apparent from this study that leukemia virus-induced immunosuppression may not be general for all anti-

TABLE 4. Effect of prior FLV infection on number of foci per splenic area in response to *V. cholerae* immunization

Expt.	FLV infection*	PFC/spleen	No. of active areas/spleen	No. of foci/area†
I	—	1,270 (170–4,660)‡	2.0	8.2 (2.3–13.0)
	+	1,010 (348–1740)	1.5	3.3 (2.0–4.6)
II	—	4,500 (1,540–11,500)	2.7	10.1 (3.8–13.7)
	+	3,892 (891–13,500)	3.2	4.1 (3.2–8.3)

* Mice were infected with 1–5 ID₅₀ of FLV, 3 or 4 days before immunization.

† Average number of foci and range calculated from 8 to 32 areas for groups of six to eight mice.

‡ Average number of PFC, with range (six to eight mice).

gens, but selective. The data presented here indicate that susceptibility of immune response to leukemia virus-induced immunosuppression is related to the functional status of an immunocompetent antigen-reactive cell, rather than to immunologic specificity. Thus, the experimental model of FLV infection provides a promising tool to distinguish between nonsensitized cells and memory-cell clones. It could be expected on the basis of the experiments presented here that even the antibody response to sheep erythrocytes would be unaffected by FLV infection in germfree animals deprived of natural sensitization and lacking an immune "background." It has already been reported (13) that Rauscher leukemia virus replicates more vigorously in the spleen of germfree mice immunized previously with sheep erythrocytes than in nonimmunized germfree controls. This finding also supports the view that a previously stimulated lymphoid cell may be more susceptible to virus infection. On the other hand, antigen-stimulated cells that have already fully developed the antibody synthesizing activity are not suppressed by FLV (7, 14). Thus, it may be predicted that the period of susceptibility to virus is limited to a specific step of lymphoid-cell differentiation. This phenomenon may be more general and may also involve interaction of nononcogenic leukemia viruses with lymphoid cells; viruses such as Vesicular Stomatitis or Newcastle Disease virus replicate *in vitro* in antigenically sensitized lymphocytes, but not in nonsensitized ones (15). The effects of other murine leukemia viruses such as Gross and Moloney virus are being studied in the cholera model system in this laboratory.

The significance of the slight but consistent increase in the antibacterial response during leukemogenesis is difficult to explain. Nevertheless, it seems that a particular antigen-reactive cell may replicate together with other cells in the enlarging spleen of an infected mouse. However, an immunoenhancing effect by lactate dehydrogenase virus, which often contaminates Friend-virus preparations, cannot be excluded (16, 17).

Indirect evidence indicates that the splenic foci of PFC may be created by immunologically specific antigen-reactive cells that differentiate and proliferate into clonal-like formations of antibody-forming cells (11, 12). This normal function may be suppressed by FLV infection. However, PFC accumulate in the spleen at the same rate. This suggests the interesting possibility that the normally occurring differentiation of antigen-reactive cells to PFC after primary antigenic stimulation may be only a facultative process that is susceptible to diversion to another developmental pathway in the process of leukemogenesis.

This discussion may be relevant to the problem of immunization of an individual against a leukemia virus. If virus

infection is accompanied by immunologic sensitization of specific antigen-reactive cells to virus antigen, followed by subsequent inhibition, the possibility of effective immunotherapy would be remote. On the other hand, if the virus first establishes immunological tolerance toward itself or neoantigens (18), and such tolerance is induced without concomitant immunological sensitization—as has been shown in other experimental systems (19)—the possibility of reversing tolerance and immunizing the individual would be theoretically possible.

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