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IMMUNOSUPPRESSIVE ACTIVITY OF ANTIBODY DIRECTED AGAINST ENDOGENOUS C-TYPE VIRUS INTERFERES WITH EARLY EVENTS OF THE IMMUNE RESPONSE

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We have analyzed the effects of an antiserum prepared against BALB/c endogenous xenotropic C-type virus on the humoral immune response of mice. Both *in vivo* and *in vitro*, this serum suppresses the response to sheep red blood cells, an effect that can be absorbed out by purified BALB/c xenotropic C-type virus or Friend leukemia virus, but not by Rous sarcoma virus. The serum produces its maximum effect when administered together with or before the antigen, but not 24 hr later. This suggests that it acts on an early event of the immune response. Evidence is presented to show that the critical viral antigen is expressed before the spleen cells are experimentally stimulated by antigen. The same immunosuppressive effect was observed in a variety of mouse strains, including the high-leukemia incidence AKR strain and virus-free 129/J mice, indicating that it is independent of the expression of endogenous virus. The finding that a viral antigen is involved in the transition from a resting to a dividing lymphocyte is discussed with respect to viral involvement in leukemia.

During the last decade, a growing body of information has accumulated from which it is evident that in a variety of species RNA C-type viruses occur endogenously as convalently integrated DNA proviruses. Different explanations have been offered to account for the presence of these viral genes and their maintenance in evolution. According to one hypothesis, viral gene products, e.g., reverse transcriptase, are involved in physiologic host functions (1). An alternative explanation based on the replication cycle of these RNA viruses and not ascribing any physiologic role to proviral genes has been put forward by Baltimore (2). A third hypothesis, the virogene-oncogene hypothesis, states that virus expression occurs in carcinogenesis (3).

We have investigated whether endogenous virus may play a physiologic role in the immune system. This possibility was suggested by earlier observations of our own (4–9) and of others (10–12) that various polyclonal B cell activators, which in some respects are thought to mimic physiologic antigenic stimulation, lead to the induction and release of endogenous xenotropic C-type virus in mice. Our approach consisted of testing the effect of antiserum directed against BALB/c endogenous xenotropic virus on the humoral immune response. We reported recently that this antiserum suppressed the humoral immune response of BALB/c mice to sheep red blood cells (SRBC) both *in vivo* and *in vitro*. Absorption studies confirmed the viral specificity of this effect (13). In this report we present evidence that the immunosuppressive activity interferes with the early events of the immune response. Furthermore, we show that this phenomenon is not restricted to mouse strains capable of expressing complete endogenous C-type virus.

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MATERIALS AND METHODS

Mice. Specific pathogen-free BALB/c, AKR, DBA_2, C57BL and $(C57BL \times DBA_2)F_1$ mice were purchased from Bomholdgard, Denmark, and 129/J mice from Jackson Laboratories, Bar Harbor, Maine. The animals were used at 8 to 12 weeks of age.

Preparation of antiviral sera. The endogenous xenotropic BALB/c virus used for immunization was originally induced in BALB/c spleen cell cultures by lipopolysaccharide *Escherichia coli*, as previously described (4, 8). Infection of rabbit SIRC cells with this virus resulted in a permanent virus-releasing cell line. The virus was purified by sucrose density gradient centrifugation from culture supernatants. For immunization, rabbits were repeatedly injected intramuscularly with doses of about 0.2 mg protein emulsified with complete Freund's adjuvant. Preimmunization sera from the same animals were used for controls. The sera were inactivated by heating at 56°C for 30 min, sterilized by Millipore filtration, and stored at −70°C. The antisera selected for these studies displayed C-dependent cytotoxicity on virus-infected SIRC cells (titers 1:40 to 1:80) but did not react with uninfected SIRC cells as shown by cytotoxic test and fluorescence assay. Control sera were negative on infected as well as uninfected SIRC cells.

In vitro immunization. An antibody response to SRBC was induced in spleen cell cultures by the Mishell-Dutton technique (14). 8×10^6 spleen cells were cultured in 35- x 10-mm disposable tissue culture Petri dishes (Falcon, Oxnard, Calif.) containing 1 ml RPMI 1640 medium (Microbiological Associates, Bethesda, Md.) supplemented with 8% fetal bovine serum (Rehatuin, Reheis, Chicago, Ill.), 1% horse serum (Microbiological Associates) and antibiotics. 4×10^6 SRBC were added at the beginning of the 5-day culture period. The Petri dishes were rocked and maintained in an atmosphere of 10% CO_2, 83% N_2 and 7% O_2. At various intervals, 10 μl of serum dilution were added. Direct plaque-forming cells (PFC) were assayed by the local hemolysis technique in liquid medium (15).

In vivo immunization. Mice were immunized by a single i.p. injection of 5×10^8 SRBC. Antiserum or control serum (0.2 ml) was injected i.v. at the time of antigen application or as indicated. Spleen PFC were assayed 4 days after immunization (15).

RESULTS

The results of antiserum directed against BALB/c endogenous xenotropic C-type virus on the *in vitro* immune response is shown in Table I. The antiserum produced a dose-dependent immunosuppressive effect, reducing the number of PFC by 86% at a 1:100 dilution (Table I, expt. 1). The immunosuppressive property of the rabbit antiserum was removed by prior absorption with xenotropic BALB/c virus grown in mink lung cells and with Friend leukemia virus grown in mouse cells, but not with Rous sarcoma virus. The absorption was found to be dose-dependent (Table I, expts. 2 and 3). The possibility that the immunosuppressive activity was due to antibodies directed against fetal bovine serum components was considered since fetal bovine serum was present both in the cultures from which viruses used for immunization and absorption were grown as well as in Mishell-Dutton cultures. However, absorption with fetal bovine serum had no effect (Table I, expt. 3). Furthermore, extensive absorption with uninfected rabbit SIRC cells grown in the presence of fetal bovine serum had no effect (data not shown). In these 5-day cultures, antigen-sensitive precursor B cells are triggered to divide and differentiate into plaque-forming end cells. The antiviral serum was added on various days to determine whether it interfered with early or late events of the immune response. Table II shows that the antiserum caused maximum inhibition when it was present from the start of culturing, but lost its effectiveness when added 24 hr later. This suggests that the antibody interferes with an early part of the immune response.

These observations were confirmed in *in vivo* experiments. Mice were injected with the antiviral serum before, simultaneously with, or after immunization with SRBC. Again, the effectiveness of the serum was reduced when it was injected 1 or more days after immunization, whereas the same degree of immunosuppression was observed when the antiserum was injected 1 or 2 days before immunization (Table III). To determine whether the critical antigen involved in this effect was exposed before SRBC recognition, BALB/c spleen cells were incubated for 45 min with antiserum or control serum, then extensively washed and used for Mishell-Dutton cultures. Table IV shows that spleen cells that had been incubated with antiserum were immunosuppressed indicating that the responsible antigens were expressed before triggering by SRBC occurred.

TABLE I

In vitro immunosuppression by antiserum directed against endogenous xenotropic BALB/c virus

Expt. No.	Antiserum[a] (Final Dilution)	Absorbed with[b] (μg of Virus Protein)	PFC/10^7 Spleen Cells[c]	Inhibition
				%
1			5800	
	1:100		800	86
	1:400		1600	72
	1:1600		3200	45
	1:6400		4600	21
2			2700	
	1:1000		500	81
	1:1000	x-BALB[d] (25)	900	67
	1:1000	x-BALB (100)	1300	52
	1:1000	x-BALB (400)	2300	15
3			4600	
	1:1000		1500	67
	1:1000	FLV[e] (50)	1500	67
	1:1000	FLV (200)	3100	33
	1:1000	FLV (400)	4100	11
	1:1000	RSV[f] (400)	1800	61
	1:1000	FBS[g]	1900	59

[a] Ten microliters of diluted antiserum, directed against BALB/c endogenous xenotropic C-type virus, were added at the onset of the cultures.

[b] Virus purified on a sucrose density gradient was pelleted by centrifugation. For absorption, the pellet was resuspended in 30 μl of antiviral serum, diluted 1/10, and incubated for 45 min on ice. Serum was recovered by centrifugation for 60 min at 30,000 × G and added in 10-μl aliquots to the 1-ml Mishell-Dutton cultures.

[c] Direct plaque-forming cells secreting immunoglobulin against SRBC were determined 5 days after stimulation of BALB/c spleen cell cultures with SRBC. Cell recovery from various cultures did not differ significantly. The values shown are the means of triplicate determinations.

[d] Mitogen-induced endogenous xenotropic BALB/c virus, grown in mink CCL-64 cells.

[e] Friend leukemia virus, grown in mouse Evelyn cells.

[f] Rous sarcoma virus (kindly provided by Dr. K. von der Helm).

[g] Thirty microliters of antiserum (1:10) was incubated for 45 min on ice with 30 μl of FBS (fetal bovine serum) followed by centrifugation at 30,000 × G and added in 20-μl aliquots to the 1-ml Mishell-Dutton cultures.

TABLE II

Time dependency of the immunosuppressive effect in vitro

Serum Addition[a]		PFC/10^7 Spleen Cells[b]	Inhibition
	day		%
None		9100	
Control serum	0	8700	4
Antiserum	0	3700	59
Antiserum	1	8000	12
Antiserum	2	8500	7
Antiserum	3	8800	3
Antiserum	4	8200	10

[a] Final dilution of serum 1/1000.

[b] Same conditions as described in Table I, footnote *c*.

TABLE III

Time dependency of the immunosuppressive effect in vivo[a]

Serum Injected (Dilution)	Injection	PFC/10^7 Spleen[b] Cells ± S.E.M.	Inhibition
	day		%
		17,300 ± 100	
Control serum (1/5)	0	17,900 ± 200	−4
Antiserum (1/5)	−2	5,900 ± 300	66
	−1	6,000 ± 100	65
	0	6,100 ± 100	65
	1	10,500 ± 200	39
	2	14,300 ± 100	17
	3	14,700 ± 200	15
	4	17,200 ± 100	1

[a] BALB/c mice were immunized on day 0 by i.p. injection of SRBC. Antiserum or control serum (0.2 ml) was injected i.v. on the days indicated. On day 4, animals were killed and the number of direct PFC determined.

[b] Average of quadruplicate values from spleens of two individual mice.

Finally, we asked whether our antiviral serum was immunosuppressive for mice other than the BALB/c strain. We included strains that naturally express ecotropic virus *in vivo* (AKR), strains with virtually no virus expression as young adults (C57BL/6, BALB/c, DBA_2), as well as the 129/J strain, from which no C-type virus has ever been isolated. The data presented in Table V show that all strains tested could be immunosuppressed by the antiviral serum, both *in vivo* and *in vitro*.

DISCUSSION

The terminal differentiation of antigen-sensitive B cells is a complex process, triggered by the binding of antigen to the immunoglobulin receptor on the B cell membrane and generally requiring cell-cell interaction with helper T cells and macrophages. Our results suggest that antiviral serum blocks some early step(s) in this complex chain of events, possibly the triggering event itself. They indicate that the immune response, once triggered by antigenic stimulation, loses its sensitivity to the antiviral serum. Although we know now that the critical viral antigen is exposed before antigenic stimulation, we have no information yet on its biochemical nature. What could be the mechanism of this immunosuppression? It might be that the serum lyses cells participating in the immune response in a C-dependent way, but this seems unlikely since we and other investigators could not demonstrate lytic C activity in Mishell-Dutton cultures (unpublished results and 16). We propose that binding of antibody to viral antigen on the cell membrane either leads to steric hindrance of immunologic receptor structures, or, alternatively, that viral antigen itself plays a receptor-like role: it could, for example, be involved in cell-cell recognition. Antisera that also block the immune-response include anti-immunoglobulin serum (17) and anti-Ia serum (18, 19). It will be interesting to elucidate the physical and chemical relationship between viral antigen, Ia-membrane antigen and immunoglobulin receptor.

The fact that viral antigen is exposed before antigenic stimulation is surprising in view of our earlier findings that nonspecific polyclonal activation of B cells leads to the expression of mature xenotropic virus 2 to 3 days after stimulation (4, 8). Further work is required to clarify the relationship between the suppression of B cell immunity reported here and the expression of infectious endogenous virus after mitogenic stimulation. However, supportive evidence for the expression of viral antigen after antigenic stimulation has recently been presented by Wecker *et al.* (16). Using C-dependent cytotoxicity they showed expression of the viral gp70 glycoprotein at the level of helper T cells and plaque-forming B cells but not precursor cells.

If the serologic effect analyzed in this paper reflects some physiologic role of endogenous viral antigens in the immune response, the effect should be found in a variety of mouse strains, irrespective of their pattern of virus expression *in vivo* and also with antigens other than SRBC. We have demonstrated the immunosuppressive effect *in vivo* and *in vitro* for strain 129/J, from which no endogenous virus has ever been induced but which carries xenotropic viral sequences (S.K. Chattopadhyay, personal communication) as well as for mice that express ecotropic endogenous virus throughout most of their lives, e.g., AKR. Furthermore, the immune response to a

TABLE IV

In vitro immunosuppression after preincubation of spleen cells with serum and washing[a]

Expt. No.	Cells Preincubated with	PFC/10^7 Spleen Cells	Inhibition
			%
1		3150	
	Control serum	3650	−16
	Antiserum	450	86
2		4100	
	Control serum	3800	7
	Antiserum	1400	66

[a] Fifty microliters of antiserum or control serum were incubated together with 4×10^7 BALB/c spleen cells in a total volume of 1 ml for 45 min at 37°C. The cells were then washed once (Expt. 1) or twice (Expt. 2) in 40 ml of RPMI 1640 medium and assayed in the Mishell-Dutton system, as described in Table I.

TABLE V

Immunosuppressive effect in different strains of mice

Strains	Antiserum	*In Vitro*[a]		*In Vivo*[b]	
		PFC/10^7 Spleen Cells	Inhibition	PFC/10^7 Spleen Cells	Inhibition
			%		%
BALB/c	−	4000		25,000 ± 1,000	
	+	900	77	4,800 ± 300	81
AKR	−	1700		11,800 ± 300	
	+	400	76	4,600 ± 300	61
DBA_2	−	7300		6,600 ± 600	
	+	300	96	1,400 ± 100	79
C57BL	−	1900		25,200 ± 1,500	
	+	700	63	1,600 ± 400	94
(C57BL × DBA_2)F_1	−	2100		15,000 ± 700	
	+	500	76	4,200 ± 400	72
129/J	−	2400		17,800 ± 400	
	+	600	75	4,600 ± 700	74

[a] See Table I, footnote 3. Final antiserum dilution, 1/200.

[b] After immunization with SRBC, mice received an intravenous injection of 0.2 ml antiserum or control serum (diluted 1/2). Plaque-forming cells were determined on day 4. The figures quoted are averages of quadruplicate values from two individual mice.

soluble antigen, namely trinitrophenylated keyhole limpet hemocyanin, was also suppressed (unpublished results with Peter Erb). These data support our hypothesis that viral antigens may be physiologically involved in the immune response irrespective of mouse strain or antigen.

The basic similarity between a resting lymphocyte triggered to give rise to a clone of dividing cells and a normal lymphocyte transformed to a malignant leukemic cell is clearly the commitment to proliferation. Should endogenous viruses play an active role in the triggering of lymphocytes, it may be possible to find a link between this system and leukemias where C-type viruses are known to play an as yet undefined role. Understanding the function of endogenous viruses in physiologic cell activation may then illuminate the viral role in leukemogenesis.

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