INDUCTION AND CHARACTERIZATION OF SPLENIC SUPPRESSOR CELLS DIRECTED TO NATURAL KILLER CELLS IN JAPANESE QUAILS

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SUMMARY: Intravenous inoculation of chicken amniotic fluid (ChAmF) markedly reduced natural killer (NK) cell activity of spleen cells from Japanese quails. The reduction of NK activity was mediated by non-adherent thymus-dependent lymphoid cells which were resistant to treatment with anti-immunoglobulin serum and sensitive to treatment with anti-thymocyte serum in the presence of complement. The suppressing activity was selectively directed to NK cells, since Rous sarcoma virus-specific cytotoxicity or hemagglutinating antibody production against sheep erythrocytes was not suppressed in ChAmF-treated quails. Spleen cells from normal 1-week-old quails had similar characteristics to those from ChAmF-treated 4-week-old quails, lacking NK activity and exhibiting suppressive effect on NK activity, and were also shown to be thymus-dependent. Biological significance of the presence of NK cells and their suppressor cells is discussed in relation to embryonic development and tumor-surveillance mechanism.

INTRODUCTION

It has been well established that various types of suppressor cells, i.e., suppressor T cells (Basten, Loblay and Pritchard-Briscoe, 1977), B cells (Neta and Salvan, 1976) and macrophages (Kirchner, Fernbach and Herberman, 1976) are involved in the regulation of diverse immune responses. As regards the natural killer (NK) cells, little information is available on their regulation by suppressor cells. A few reports demonstrated suppressor cells directed to NK cells (Savary and Lotzva, 1978; Hochman and Cudkowicz, 1979; Santoni et al., 1978).

In a previous paper (Yamada et al., 1980), we demonstrated the presence of NK cells in the spleens of Japanese quails and induction of suppressor cells directed to NK cells by treating quails with chicken amniotic fluid. In the present study, the properties of these suppressor cells including their specificity and sensitivity to several treatments were examined.

MATERIALS AND METHODS

Animals: Japanese quails (*Coturnix coturnix japonica*) were obtained from the Nippon Institute for Biological Science, Tokyo, and used at the age of 4 to 8 weeks unless otherwise specified.

Virus and cells: Schmidt-Ruppin strain of subgroup A of Rous sarcoma virus (SR-RSV) was used to infect Japanese quails. Normal quail embryo (nQE) cells, quail embryo cells transformed with SR-RSV (SR-QE), and established quail cell line, R(−) were used. R(−) cells were transformed with defective Bryan high-titer strain of RSV lacking gp85, the major envelope glycoprotein.

Treatment of quails with chicken amniotic fluid (ChAmF): Quails were treated with ChAmF of 8-day-old embryos as described previously (Yamada et al., 1980). In brief, quails received four *iv* inoculations each with 0.2 ml of ChAmF at 2-day intervals and their spleen cells were assayed for NK activity 2 days after the last injection.

Assay for NK activity: NK activity was measured as described before by the microcytotoxicity test (Yamada et al., 1980). In brief, target cells prepared in Falcon 3034 microtest plates were incubated with a spleen cell suspension at 39 C for 16 hr, and surviving target cells were counted after staining with a 2% Giemsa solution. Percent cytotoxicity was calculated by the following equation:

$$\frac{\text{Number of cells remaining without effector cells} - \text{Number of cells remaining with effector cells}}{\text{Number of cells remaining without effector cells}} \times 100$$

Detection of suppressor cells: Normal spleen cells exhibiting NK activity were mixed with spleen cells taken from test animals and the mixture was assayed for NK activity as mentioned above.

Production of hemagglutinating (HA) antibody to sheep red blood cells (SRBC): Quails were each intravenously injected with 0.2 ml of 10% SRBC and sera were collected on day 5 after immunization. HA titers were expressed as the reciprocals of the highest serum dilutions agglutinating 0.4% SRBC on microplates.

Treatment of spleen cells: Details of depletion of adherent cells, surface immunoglobulin-bearing cells and thymus-derived cells were described previously (Yamada et al., 1980). Adherent and non-adherent cells were separated by incubating a spleen cell suspension on a plastic dish. Non-adherent cells were further treated with anti-quail immunoglobulin serum (a-Ig) or anti-quail thymocyte serum (ATS) in the presence of guinea-pig complement. The cytotoxic antibody titers were determined by the microcytotoxicity test in the presence of complement and expressed as 50% effective dose calculated by the method of Behrens-Kärber. The titers of ATS and a-Ig for thymocytes were 512 and 4, respectively, and for bursal cells 16 and 2,048, respectively.

TABLE I

Influence of S/E ratios on suppressor activity

Number of effector cells	Number of suppressor cells	S/E ratio ¹⁾	% cytotoxicity on		
			nQE	R(-)	SR-QE
1×10 ⁴	0	0	43.4**** ³⁾	59.4***	49.2***
1×10 ⁴	0.25×10 ⁴	0.25	28.3** (34.8) ²⁾	30.5*** (48.7)	33.4** (32.1)
1×10 ⁴	0.5 ×10 ⁴	0.5	8.6 (80.2)	29.0*** (51.2)	25.3** (48.6)
1×10 ⁴	1 ×10 ⁴	1.0	13.7 (68.4)	23.7 (60.1)	12.4 (74.8)

1) Suppressor cell to effector cell ratio.

2) Percent suppression is shown in parentheses.

3) Statistical significance was estimated by Student's T test comparing with control (without the addition of effector cells). *** P<0.001 ** P<0.01

RESULTS

Induction of Suppressor Cells to NK Cells

As previously reported (Yamada et al., 1980), spleen cells of normal quails exhibited NK activity on various target cells of quail origin such as nQE, R(-) and SR-QE which expressed embryonic antigen(s) (E-Ag) on their surfaces (Ignjatovic et al., 1978). The NK activity was significantly reduced in quails treated with ChAmF and the reduction was mediated by suppressor cells induced by ChAmF-treatment. As shown in Table I, such a suppressive effect was manifested at suppressor to effector cell ratios (S/E ratios) of 1:1, 0.5:1 and 0.25:1. The ratio of 1:1 was employed in the following experiments.

Specificity of Suppressor Cells

To investigate whether the suppressive effect was directed to only NK cell activity, the effect of ChAmF-treatment on RSV-specific cell-mediated cytotoxicity was examined. Quails treated with ChAmF were infected with 1,000 focus-forming units of SR-RSV. Two weeks after RSV infection, quails with progressing tumor (progressor) and those with regressing tumor (regressor) were sacrificed and their spleen cells were tested for the cytotoxicity (Table II).

Regressor spleen cells without ChAmF-treatment manifested high cytotoxicity on SR-QE and R(-) cells, and low but significant cytotoxicity on nQE cells. The cytotoxicity on the formers was considered to be due to both RSV-specific immune lymphocytes and NK cells, whereas on the latters was it considered to be due to only NK cells. In progressors without ChAmF-treatment, the cytotoxicity on SR-QE and R(-) cells was reduced to the levels close to that of normal spleen cells. As RSV-specific cytotoxicity was not detected in progressor lymphocytes (Hayami et al., 1972), such a low level of the cytotoxicity detected in progressor spleen cells was considered to be mediated by NK cells. This was confirmed by the finding that spleen cells from the progressors treated

TABLE II

Effect of ChAmF-treatment on RSV-specific cell-mediated cytotoxicity

ChAmF-treatment	RSV-immunization ¹⁾		% cytotoxicity on		
			nQE	SR-QE	R(-)
+	+	Prog. ²⁾	-14.2	3.8	7.7
+	+	Reg. ³⁾	-19.3	77.9**** ⁴⁾	51.2****
-	+	Prog.	19.1*	35.5***	16.0*
-	+	Reg.	17.9*	55.3***	42.7***
-	-		17.2	23.3*	28.3***
+	-		- 0.2	9.9	19.8

1) Spleen cells were obtained from quails immunized with 1,000 FFU of SR-RSV 2 weeks before and assayed for their cytotoxicity by the micro-cytotoxicity test.

2) Progressor spleen cells.

3) Regressor spleen cells.

4) Statistical significance was estimated by the Student's T test.

***P<0.001 *P<0.05

TABLE III

Hemagglutinating antibody titers to SRBC¹⁾

Quails	Positive ratio	HA titers
ChAmF-treated	5/5 ²⁾	32, 128, 128, >256, >256
Untreated	5/5	128, 32, 16, 128, 64

1) Sera were collected from quails immunized with 10% SRBC 5 days before. Hemagglutinating (HA) antibody titers were expressed as the reciprocals of the highest dilutions agglutinating 0.4% SRBC on microplates.

2) Number positive/Number tested.

with ChAmF showed no cytotoxicity on SR-QE and R(-) cells or nQE cells. On the other hand, spleen cells of regressors with ChAmF-treatment showed high cytotoxicity on SR-QE and R(-) cells but not on nQE cells. In vitro experiments revealed that the cytotoxicity of RSV-immune spleen cells on SR-QE or R(-) cells was not suppressed even after the addition of spleen cells from ChAmF-treated quails, whereas that on nQE cells was suppressed (data not shown). These findings suggest that ChAmF-treatment suppresses only NK cell activity but not antigen-specific cytotoxic responses.

Moreover, when HA antibody titers against SRBC in ChAmF-treated quails were compared with those in untreated quails, it is evident from Table III that the production of antibodies was not suppressed but rather slightly enhanced by treatment with ChAmF. These findings suggest that suppressor cells induced by treatment with ChAmF in quails manifest suppressive effect selectively on NK cell activity.

TABLE IV

NK activity of spleen cells from ChAmF-treated quails after various treatments¹⁾

Spleen cells	% cytotoxicity on nQE	
	Exp. 1	Exp. 2
Unfractionated	— 13.0	2.3
Adherent	1.2	13.2
Non-adherent	— 9.5	2.4
ATS-treated non-adherent ²⁾	33.6**	35.4***
a-Ig-treated non-adherent ³⁾	— 6.0	— 0.5
NRS-treated non-adherent ⁴⁾	— 4.6	8.9

1) Spleen cells of ChAmF-treated quails were separated into adherent and non-adherent cells. Both fractions were assayed for their cytotoxicity. Non-adherent cells were further processed by several treatments and then assayed for the cytotoxicity.

2) Anti-thymocyte serum plus complement.

3) Anti-immunoglobulin serum plus complement.

4) Normal rabbit serum plus complement.

*** $P < 0.001$ ** $P < 0.01$ (compared with the value obtained without the addition of effector spleen cells)

Characterization of Suppressor Cells

To determine the cell types displaying suppressive effect on NK cells, we examined the effect of several treatments on the function of ChAmF-induced suppressor cells. First, spleen cells from ChAmF-treated quails, which had no cytotoxic activity, were separated into adherent and non-adherent cells. Both fractions showed no apparent cytotoxicity, but non-adherent cells exerted NK activity after treatment with ATS in the presence of complement. On the other hand, depletion of surface immunoglobulin-bearing cells from ChAmF-treated spleen cells by treatment with a-Ig and complement failed to manifest NK activity. These results suggest that the suppressor cells were non-adherent, thymus-dependent cells (Table IV).

To confirm this finding that the suppressor cells were thymus-dependent, cell mixture experiment was performed (Table V). Spleen cells from normal quails showing NK activity were mixed with various preparations of spleen cells of ChAmF-treated quails. As shown in the table, the addition of adherent cells or ATS-treated non-adherent cells taken from ChAmF-treated quails did not eliminate the cytotoxic activity of normal spleen cells. No other treatments interfered with suppressive effect of ChAmF-treated spleen cells and NK activity of normal spleen cells was suppressed when they were mixed. It was concluded that suppressor cells are thymus-dependent non-adherent lymphoid cells.

TABLE V

NK activity of normal spleen cells after mixing with various preparations of spleen cells taken from ChAmF-treated quails

Effector	Preparations of spleen cells of ChAmF-treated quails	% cytotoxicity on	
		nQE	R (-)
Normal spleen cells	—	43.5***	34.4***
Normal spleen cells	Unfractionated	13.0*	8.3
Normal spleen cells	Adherent	36.0***	47.8***
Normal spleen cells	Non-adherent	5.0	14.7*
Normal spleen cells	ATS-treated non-adherent	33.3***	45.5***
Normal spleen cells	a-Ig-treated non-adherent	10.1	4.8
Normal spleen cells	NRS-treated non-adherent	6.8	4.9

*** $P < 0.001$ * $P < 0.05$ (compared with the value obtained without the addition of effector cells)

TABLE VI

NK activity of spleen cells from 1-week-old quails

Spleen cells taken from quails aged (in weeks)	Treatment	% cytotoxicity ¹⁾
1	—	6.2
4	—	33.3***
1+4 ²⁾	—	6.4
1	ATS+C ³⁾	39.1***

1) R(-) cells were used as target cells.

2) After spleen cells from 1-week-old quails and from 4-week-old ones were mixed at a ratio of 1:1, the cytotoxicity of the mixture was assayed.

3) Anti-thymocyte serum plus complement

*** $P < 0.001$ (compared with the value obtained without the addition of effector spleen cells).

Suppressor Cells in the Spleens of 1-week-old Quails

In the previous report (Yamada *et al.*, 1980), NK activity was not demonstrated in spleen cells from 1-week-old quails. As shown in Table VI, the lack of NK activity in infant birds was due to the presence of suppressor cells, since the addition of spleen cells from 1-week-old quails to those from 4-week-old ones reduced the cytotoxicity of the latters. Moreover, NK activity of spleen cells from 1-week-old quails became detectable after the treatment with ATS and complement. This indicates that the treatment with ATS eliminated ATS-sensitive suppressor cells from spleen cells of 1-week-old quails. Thus the presence of suppressor cells resembling those of ChAmF-treated quails but not absence of NK cells was suggested as the cause of the lack of NK activity in infant quails.

DISCUSSION

By inoculating ChAmF into Japanese quails, we induced thymus-dependent suppressor cells which selectively inhibited NK activity manifested by normal quail spleen cells.

Recently the demonstration of suppressor cells of which effect is directed to NK cells has been documented by several investigators. Savary and Lotzva (1978) reported the presence of suppressor cells for NK cells in the spleens of infant mice, *Corynebacterium parvum*-injected mice and bone marrow-tolerant mice, however no further characterization of these suppressor cells was informed. Hochman and Cudkowicz (1979) induced suppressor cells for NK cells by treating mice with hydrocortisone and showed that cells responsible for the suppression had extraordinary characteristics in that they could be removed by carbonyl iron but not by other adherence techniques. More recently, it was shown that splenic NK activity was transiently suppressed by activated macrophages in adriamycin-treated mice (Santoni et al., 1980). Thus most of these studies suggested a possible involvement of cells of macrophage lineage as the suppressors. In contrast, the present study disclosed that suppressor cells induced by treatment with ChAmF were thymus-derived lymphoid cells, since their effect was eliminated by treatment with ATS and complement. It seems of interest that NK cells might be regulated not only by macrophage series but also by T cell lineage. Suppressor T cells described here have a unique property in comparison with so-called suppressor T cells (Basten et al., 1977; Gershon, 1974) found in mammals in that they are induced by ChAmF and that their action is directed to NK cells selectively. It can be considered that they also differ from suppressor cells suppressing RSV-specific cellular cytotoxicity detected in Japanese quails (Hayami et al., 1972; Bauer, Hayami and Stehfen-Gervinus, 1979).

In the previous paper (Yamada et al., 1980), NK cells were suggested to exert their cytotoxic activity to embryonic antigen(s) (E-Ag) on the target cells. The inhibition of NK activity by the administration of ChAmF may indicate a possibility that E-Ag present in ChAmF directly blocked NK activity rather than by the induction of suppressor cells. This possibility, however, seems unlikely, since the addition of ChAmF to normal spleen cells in vitro did not inhibit NK cell activity of these cells (unpublished data).

Although the mechanism of suppression is still an open question, we consider that the suppression might occur in the effector phase, since a normal level of NK activity was recovered in spleen cells from which suppressor cells were eliminated by treatment with ATS and complement (Table IV). Whether the suppression is manifested by direct cell-to-cell interaction or indirectly by soluble factors as indicated in other systems (Kontinen and Feldmann, 1977; Tada, Taniguchi and David, 1976) is a subject for further analysis.

Since E-Ag is demonstrated both in embryonic cells and tumor cells (Ignjatovic et al., 1978; Yoshikawa, Ignjatovic and Bauer, 1979), E-Ag-specific

NK cells and their suppressor cells may be teleologically speculated to participate in both normal embryonic development and tumorigenesis. The presence of suppressor cells at embryo and infant stages may protect the normal embryonic cells from the destruction by NK cells, whereas NK cells may exert cytotoxic activity by recognizing E-Ag expressed on the surface of tumor cells in adult hosts in which suppressor cells are absent.

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