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Studies on induction and control of cell-mediated autoimmunity

I. Induction of "autoreactive" T lymphocytes in mice by cyclophosphamide

Injection of a single dose of cyclophosphamide (CY) (125 mg/kg) or a combination of a small dose of CY (20 mg/kg) and 2.5 mg/kg lipopolysaccharide induces a transient appearance of autoreactive T lymphocytes (T-ARC) in the spleens of mice. The T-ARC activity reaches a peak 6 days after CY injection and could not be detected 8 days after this treatment. For testing T-ARC activity, spleen cells were injected into the footpads of syngeneic recipients, and the resulting lymph node enlargement at the draining site of cell inoculation and the content of nucleated cells in the lymph node was determined. Possible explanations of this autoimmune phenomenon are discussed. It is postulated that CY-resistant precursors of T-ARC are stimulated by "new" antigenic sites present on the surface of B lymphoblasts repopulating the CY-damaged spleen in a period of transient absence of CY-sensitive suppressor cells.

1 Introduction

There is increasing evidence of potentially autoreactive cells (ARC) in normal animals [1, 2]. Expression of autoimmunity, therefore, may not arise primarily as an effector cell abnormality but rather as a consequence of a defect in a thymus-dependent control mechanism [3, 4].

It has been reported [5] that adult thymectomy promotes the manifestation of autoreactive lymphocytes as tested *in vivo* by graft-*vs.*-host (GvH) reactions. As a possible explanation for this phenomenon, the hypothesis was made that thymus ablation induces the progressive loss of suppressor T cells permitting the manifestation of ARC.

Cyclophosphamide (CY) enhances the capacity of mice to give T cell-dependent responses such as delayed-type hypersensitivity (DTH) to sheep red blood cells [6]. Askenase et al. [7] presented results indicating that CY may preferentially deplete the mice of suppressor T cells involved in the negative control of DTH. Similar conclusions have been drawn by Röllinghoff et al. [8], who observed induction of cytotoxic lymphocytes in mice to hapten-conjugated syngeneic cells after CY treatment. Thus, if one accepts that ARC (or their precursors) exist in normal mice and that CY may deplete suppressor cells or their precursors, treatment of normal mice with CY may lead to the manifestation of ARC.

Preliminary experiments [9, 10] indicated that a transient impairment of a thymus-dependent regulatory mechanism in normal mice is followed by the appearance of a cell-mediated autoimmune phenomenon. In this report, we describe the conditions for induction and detection of ARC *in vivo* and describe some properties of these cells.

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Abbreviations: ARC: Autoreactive cells **T-ARC:** Autoreactive T cells **LN:** Lymph node **GvH:** Graft-*vs.*-host reaction **CY:** Cyclophosphamide **DTH:** Delayed-type hypersensitivity **LPS:** Lipopolysaccharide of *E. coli* **ATx:** Adult thymectomy (thymectomized) **SEM:** Standard error of the mean **ITL:** Initiator T lymphocytes

2 Materials and methods

2.1 Treatment of mice

Female BALB/c or ARK mice (from Gl. Bomholtgård, Ry, Denmark) were used at the age of 6–10 weeks. CY (Endoxan, Asta-Werke, Brackwede, FRG) was dissolved in sterile saline, and a volume of 0.5 ml containing the appropriate dose of CY was injected intraperitoneally (i.p.) within 30 min after dis-

solving. LPS (lipopolysaccharide from *E. coli* 055 B5 obtained from Difco, Detroit, MI) was dissolved in saline, and 2.5 mg/kg injected i.p. in a volume of 0.5 ml.

2.2 Spleen cell preparations containing ARC

Five to 9 days after a single dose of CY (125 mg/kg) the spleens were removed under sterile conditions. Single cell suspensions were prepared in RPMI-1640 medium (Gibco, Grand Island, NY), supplemented with 5 % heat-inactivated fetal calf serum (Seromed, München, FRG), penicillin (100 IU/ml) and streptomycin (100 μg/ml) as described in detail earlier [10]. Nylon wool-nonadherent cells were prepared according to Julius et al. [11]. The cells recovered from the nylon wool columns were washed and resuspended in RPMI-1640 medium without fetal calf serum.

2.3 Anti-Θ treatment of ARC

In some experiments, nylon wool-filtered AKR spleen cells were divided into two batches, each containing 1 x 10^7 cells/ml. One batch was incubated with normal serum from C3H mice (final concentration 1:40) and a 1:5 dilution of agarose-absorbed guinea pig complement (from Biologische Arbeitsgemeinschaft, D-6202 Lich, FRG), the other batch of cells was incubated with a 1:40 dilution of C3H anti-AKR Thy-1.1 serum (from Searle Diagnostics, High Wycombe, GB) and complement. The cell suspensions were incubated at 37 °C for 45 min, washed 3 times and resuspended in equal volumes of serum-free RPMI-1640 medium.

2.4 X-irradiation of ARC-containing cell suspensions

Splenic T cells (1.2 x 10^7 cells/ml RPMI medium containing 15 % fetal calf serum) were irradiated with 1200 rad in sterile petri dishes using a Siemens x-irradiation source (Stabilipan) delivering 89.4 rad/min. After irradiation, the cells were washed in serum-free medium, resuspended in RPMI medium and 50 μl medium containing equal numbers of irradiated or control cells, and injected into syngeneic recipients.

2.5 Popliteal lymph node (LN) assay

Usually, 3 x 10^6 spleen or "splenic T cells" (nylon wool-nonadherent, Thy-1.1 -positive spleen cells) from control or from CY-pretreated mice (various times after injection of either CY, LPS or a combination of both) in a volume of 0.05 ml were injected into one hind footpad of syngeneic recipients (usually 5–7 age-matched recipients per group) using a 27 gauge needle. Six days after cell transfer, the draining and the contralateral LN were removed, and the resulting response was either calculated as the weight ratios ± SEM between the draining and the contralateral LN or as the contents of nucleated cells in the pooled LN of each experimental group.

3 Results

3.1 Induction of LN enlargement by spleen cells derived from syngeneic CY-pretreated donors

In the first series of experiments, BALB/c mice were injected with 125 mg/kg CY i.p. and spleen cells collected 6 days afterwards. Syngeneic recipients were injected into the right hind footpad with graded numbers (3 x 10^6–6 x 10^6) of spleen cells or nylon wool-filtered spleen cells from normal mice, or with spleen cells or nylon wool-filtered spleen cells derived from CY-pretreated animals. Six days later, the draining LN and the contralateral LN were removed and weighed separately. In addition, the numbers of nucleated cells in the pooled LN from each experimental group were counted. The results are summarized in Table 1.

The LN afferent to the site of injection of either spleen cells or nylon wool-nonadherent cells derived from CY-pretreated donors were significantly enlarged as compared either to the contralateral LN or to the draining LN of recipients injected with normal or nylon wool-filtered spleen cells. The increase in the numbers of nucleated cells in the LN paralleled these observations. The nylon wool-nonadherent cells still had the potency to induce LN enlargement and were considered as ARC.

Table 1. LN enlargement in syngeneic BALB/c hosts induced by injection of normal spleen cells or by cells from donors treated 6 days before with CY

Source of transferred cells	No. of transferred cells[a] x 10^{-6}	No. of recipients	LN weight[b]	LN nucleated cells[c]
Normal spleen[d]	3	16	1.16 ± 0.09	1.14
Normal spleen	6	6	1.38 ± 0.12	1.47
Nylon wool-filtered[d] normal spleen cells	3	10	1.18 ± 0.08	1.28
Spleen cells of CY-pretreated mice[e]	6	6	3.01 ± 0.32	3.80
Nylon wool-filtered spleen cells of CY-pretreated mice[e]	3	36	3.32 ± 0.17	4.43

a) Cells were injected in 0.05 ml volume into one hind footpad of syngeneic recipients.
b) Ratio ± SEM of weight of draining over contralateral popliteal LN in syngeneic hosts 6 days after cell transfer.
c) Ratio of nucleated cells in the pooled LN.
d) BALB/c mice.
e) BALB/c mice were injected with 125 mg/kg CY 6 days before the spleens were removed.

3.2 Kinetics of appearance of ARC in donor spleens

A single dose of CY (125 mg/kg) was given i.p. into a group of 30 BALB/c mice. Each day, beginning 5 days after CY treatment, 6 animals were killed, their spleens pooled, and the cell suspension filtered through nylon wool columns. These cells (3 x 10^6) were injected into the hind footpads of 5–7 syngeneic recipients and the resulting LN enlargement in these recipients determined 6 days afterwards. Cells collected from donors 6 days after CY treatment elicited up to a 5-fold enlargement of the draining LN of the recipients. Cells derived 8 days after CY treatment failed to elicit a response. As compared to BALB/c spleen cells, cells derived from AKR mice showed a significantly higher ARC activity, but the pattern of appearance and disappearance of ARC was similar in both strains.

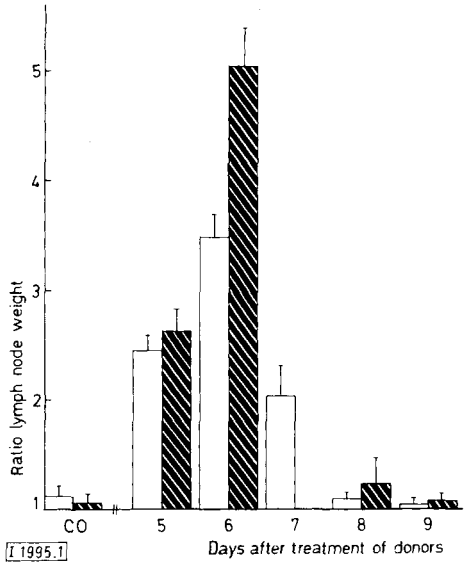

Figure 1. Kinetics of appearance and disappearance of ARC in the spleens of CY-treated donors. Mice were injected with 125 mg/kg CY and their spleens removed (6–12 spleens pooled) various days later. Nylon wool-filtered spleen cells (3×10^6) were injected into one hind footpad of 5–7 syngeneic recipients. The results are expressed as the weight ratio ± SEM of draining over contralateral LN detected in the recipients 6 days after cell transfer. BALB/c donors and recipients (white columns), AKR donors and recipients (dark columns). Controls (CO): 3×10^6 nylon wool-filtered cells of untreated BALB/c or AKR donors transferred into syngeneic recipients failed to induce an increase in the LN ratio of more than 1.2.

3.3 Susceptibility of ARC to anti-Θ serum treatment and x-irradiation

In order to test whether cells inducing the LN enlargement are Thy-1.1-bearing cells, nylon wool-filtered spleen cells collected from AKR donors 6 days after CY treatment were incubated with anti-Θ serum and complement. This treatment abolished the capacity of the remaining cells to induce LN enlargement. To test whether the LN enlargement in the syngeneic host and the increase in the number of nucleated cells in the LN was mainly due to a proliferation of the transferred cells, the donor cell suspensions were irradiated *in vitro* 1200 rads (a dose proven to inhibit mitotic activity of lymphocytes [12]). This treatment partially inhibited, but did not abolish, the phenomenon.

3.4 Synergistic action of low doses of CY and mitogenic doses of LPS to induce ARC

As recently reported [7], low doses of CY (20 mg/kg) which do not affect antibody production, could stimulate cell-mediated immune reactions (DTH) to sheep red blood cells indicating susceptibility of a T cell subset (suppressor cells) to this dose of CY. Using the same low dose of CY, spleen cells collected 6 days later only occasionally (3 out of 8 experiments) showed a small ARC activity. Cells collected from mice injected 6 days before with 50 mg LPS, failed to induce LN enlargement. However, a synergistic effect was observed when the donors were injected with a combination of 20 mg/kg CY and 50 μg LPS i.p.

4 Discussion

The local GvH reaction was originally used for detecting differences located at the level of the major histocompatibility complex [13] and is expressed by the increase in the weight of the draining LN and the number of nucleated cells therein of mice injected with allogeneic cells. The same system has also been used to assess the reactivity of "autosensitized lymphocytes" induced *in vitro* [2]. Rat lymphocytes incubated *in vitro* on syngeneic fibroblasts injected into syngeneic recipients induced enlargement of the draining regional LN with a maximum on day 6–7 after inoculation into the footpads. The same system has been used recently for detection of ARC occurring in the spleens of adult, thymectomized (ATx) mice [5]. Spleen cells obtained several months after adult thymectomy and injected into the footpads of syngeneic mice induced a small but significant enlargement of the draining LN.

As described in this report, a similar but significantly higher LN enlargement (up to 5 times over the appropriate control) could be obtained by injecting normal recipients with spleen cells derived from syngeneic mice pretreated with a single high dose of CY (Table 1 and Fig. 2). As shown in Table 1, the ARC do not adhere to nylon wool, and their activity can be eliminated from the nonadherent cell population by treatment

Table 2. Sensitivity of ARC to anti-Θ serum treatment and x-irradiation

Source of cells	Treatment of cells before transfer[a]	No. of recipients	LN weight ratio ± SEM
Normal spleen[b]	None	6	1.30 ± 0.11
Normal spleen[b]	1200 rad	6	1.15 ± 0.07
Spleen of CY-pretreated donors[a]	None	5	3.43 ± 0.39
Spleen of CY-pretreated donors[a]	1200 rad	6	2.21 ± 0.25
Spleen of CY-pretreated donors[c]	Normal C3H serum + C	6	3.22 ± 0.37
Spleen of CY-pretreated donors[c]	C3H anti-AKR Θ serum + C	6	1.21 ± 0.11

a) Cells suspended in RPMI-1640 medium (10^7 cells/ml) were either irradiated or treated with sera and complement (C) as given in Sect. 2.3 and 2.4. After these procedures, 0.05 ml of cell suspension containing 3×10^6 viable cells were injected into the foodpads of syngeneic recipients. The results are expressed as the weight ratios ± SEM of draining over contralateral LN detected in the recipient 6 days after cell transfer.
b) Nylon wool-filtered spleen cells from BALB/c mice.
c) Nylon wool-filtered spleen cells from AKR mice.

Table 3: Synergistic effect of low doses of CY and blastogenic doses of LPS on induction of ARC in AKR mice

Treatment of cell donors[a]	No. of recipients	LN weight ratio ± SEM
None	6	1.19 ± 0.08
CY (20 mg/kg)[b]	6	1.95 ± 0.49
LPS (2.5 mg/kg)[b]	5	1.36 ± 0.19
CY (20 mg/kg) +LPS (2.5 mg/kg)[b]	5	4.06 ± 0.15
CY (125 mg/kg)[b]	6	4.94 ± 0.15

a) AKR mice.
b) CY and/or LPS were injected simultaneously i.p. Nylon wool-filtered spleen cells were obtained from these animals 6 days after treatment. 3×10^6 cells were transferred into each recipient. The results are expressed as the weight ratios ± SEM of draining over contralateral LN in the recipients 6 days after cell transfer.

of the cells with anti-Θ serum and complement, showing that the ARC belong to the T lymphocyte population (T-ARC).

The question arose whether the cells detected in the spleens of CY-treated donors are ARC similar to those obtained by sensitizing lymphocytes [2] against syngeneic tissues *in vitro*, or to the autoreactive lymphocytes present in the spleens of ATx mice [5]. Before this question can be answered, one has to take into consideration the possibility of a trivial explanation. Injection of a high dose of CY damages the cells in the spleen and in other lymphoid organs. As reported previously [9], one day after injection of 125 mg/kg CY, the number of nucleated cells in the spleens is reduced to 20 % as compared to the controls. The cell damage is followed by a rapid burst of regenerative proliferation and repopulation of the spleen. This process reaches a peak 7 days after CY treatment. Therefore, a trivial explanation for the phenomenon reported here could be that certain types of spleen cells, when introduced artificially into the LN, will proliferate in this environment without any stimulus and consequently without any immunological relevance. In this case, CY would have damaged spleen cells including cells which normally control an inherent proliferative capacity of this hypothetical subset of spleen cells. After CY treatment, the cells with the inherent proliferative capacity would reappear in the regenerating spleen earlier than the cells controlling the rate of division of the former. On the other hand, the same argument would be valid for an immunological process, if we introduce instead of "spleen cells with inherent proliferative capacity" the term "autoreactive cell" (ARC), and instead of "cells controlling the activity of these cells" the term "suppressor cells". The trivial explanation of the phenomenon can be ruled out by the finding that x-irradiation *in vitro* (Table 2) in order to inhibit the proliferative capacity of the inoculated lymphocytes, reduced but did not abolish their capacity to induce LN enlargement. Furthermore, recipients pretreated with CY 1–2 days before ARC injection failed to respond with LN enlargement [14]. These results suggest (a) that the increase in the number of nucleated cells in the LN cannot be mainly due to "inherent" capacity of the injected cells to proliferate in the host LN, and (b) that the immune system of the syngeneic host has to participate in this reaction. Similar host dependency of LN enlargement has been observed if allogeneic parental lymphocytes were injected into F_1 recipients [15, 16].

Livnat and Cohen [17] recently demonstrated that T cell-mediated immune reactions to allogeneic cells can be subdivided into several steps of cell to cell interactions, one of them taking place between syngeneic cells. T lymphocytes (ITL = initiator T lymphocytes) incubated on allogeneic cells, separated from the sensitizing cells and injected into the footpads of recipients syngeneic to the sensitized lymphocytes, induced an enlargement of draining LN. The LN enlargement was mainly dependent on recruitment and proliferation of T cells of host origin (RTL = recruited T lymphocytes).

In our system, several possibilities for an explanation of this autoimmune phenomenon have to be discussed:
(a) the cells repopulating the CY-damaged spleen contain T cells carrying "new" antigenic sites due to a transient expression of such membrane components on proliferating cells [18]. In this case, the term T-ARC would be misleading, since the inoculated T cells are antigens to which the syngeneic host would respond, and consequently the ARC are of host origin.
(b) T-ARC are immature T cells which repopulate the CY-damaged spleen. If so, T-ARC are potentially autoreactive T cells postulated by Jerne [19] and Lemonier et al. [20].
(c) T-ARC are CY-resistant cells which develop from their precursors as a result of a reaction to a stimulus ("new" self antigen?) present in the spleen of animals treated with either a high dose of CY or with a blastogenic dose of LPS. In this case, T-ARC and the mechanisms by which T-ARC induce LN enlargement when injected into syngeneic hosts are comparable to the alloantigen-stimulated ITL described by Livnat and Cohen as mentioned above. There are several observations which seem to argue for this possibility:
(a) the relative high resistance of T-ARC (Table 2) and of ITL [17] to x-irradiation, (b) the participation of the host immune system in the LN enlargement due to either T-ARC or ITL injection into syngeneic recipients, and (c) the identity of kinetics of the LN enlargement due to inoculation of either T-ARC [9] or ITL.

We started our experiments originally with the working hypothesis that ARC and their precursors coexist in normal animals and that depletion of suppressor cells by either thymectomy [5] or by CY would allow detection of ARC. As shown in Table 3, a small amount (20 mg/kg) of CY, shown in other systems to deplete mice of suppressor cells for cell-mediated immune responses [7], occasionally induced T-ARC. However, injection of a high dose of CY consistently induced T-ARC with high activity. Since CY injection induces a dose-dependent regenerative proliferation and blastogenesis – predominantly of B lymphocytes – the question arose whether blastogenesis is a prerequisite for T-ARC induction. The finding that the combination of a small dose of CY and a blastogenic dose of LPS acts synergistically for T-ARC induction supports this view. Furthermore, this finding is compatible with the hypothesis that T-ARC have to develop from their precursor cells as the result of stimulation by "new" antigens and that these "new" antigens may be carried by the blastoid cells. As candidates for such antigenic structure on CY or LPS-induced B lymphoblasts, cell membrane-associated components transiently expressed on these cells have to be taken into consideration [18, 21]. B cell mitogens, *e.g.* LPS, have been shown to induce the appearance of latent endogenous C-type RNA viruses at the surface of splenic B lymphoblasts [22, 23]. Some results reported here indicate that C-type virus expression may be related to the postulated "new" antigenic structures: (a) the observation (Fig. 1) that AKR mice (known to express higher amounts of endogenous ecotropic and xenotropic viruses [24]), when injected with CY develop an augmented activity of T-ARC as compared to BALB/c mice, expressing lower titers of C-type viruses; and (b) that injection of interferon inducers [25], such as double-stranded polyinosinic-polycytidylic acid or dextran sulfate – B cell mitogens which failed to induce C-type virus expression [22, 23] – even abolished development of T-ARC when given one day after a high dose of CY (J. L'age-Stehr, unpublished observation).

The cells carrying autoreactive properties show T cell characteristics, but B cell blastogenesis seems to be required for generation of T-ARC as well as for suppressor cells counteracting T-ARC activity [14]. Therefore, we would like to postulate that in our system a transient depletion of the spleen of suppressor cell precursors and B cell blastogenesis are the prerequisite for induction and demonstration of T-ARC.

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Studies on induction and control of cell-mediated autoimmunity

II. Prevention of induction and activity of autoreactive T cells by suppressor cells and by a suppressive serum factor*

Autoreactive T lymphocytes (T-ARC) can be detected in the spleen of mice treated with a single dose of cyclophosphamide (CY) (125 mg/kg), a peak occurring 6 days after CY injection. Eight days after CY treatment, the mice develop a specific anergic state. This anergic state can be transferred to normal syngeneic animals by either splenic nylon wool-nonadherent lymphocytes (suppressor cells, S-ARC) or by the serum of anergic mice, implying the development of an active suppressive mechanism due to CY treatment. Precursors of both potentially T-ARC as well as S-ARC coexist in the spleen of normal animals. Precursors of S-ARC present in the spleen and in the thymus of normal animals are sensitive to CY. However, committed S-ARC obtained from anergic mice are resistant to CY. Committed S-ARC as well as their precursors prevent induction of T-ARC. Committed S-ARC counteract expression of committed T-ARC activity, whereas precursors of S-ARC fail to do so.

The autoimmune phenomenon described here represents an *in vivo* animal model system for induction of T-ARC and for the control mechanism which normally prevents induction and/or expression of cell-mediated autoreactivity by specific suppressor cells and by suppressive factors.

[I 1996]

1 Introduction

As previously reported [1], autoreactive T cells (T-ARC) are transiently detectable in spleen cell suspensions of mice injected with a single high dose of cyclophosphamide (CY) or after injection of a small dose of CY together with a blastogenic dose of lipopolysaccharide (LPS). Maximum T-ARC activity was found 6 days after CY; however, no T-ARC activity was present 8 days after this treatment.

We investigated whether the failure to detect ARC activity in the spleen, 8 days after CY treatment, is due to the absence

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Abbreviations: **ARC:** Autoreactive cells **T-ARC:** Autoreactive T cells **S-ARC:** Suppressor cells for T-ARC **LN:** Lymph node **CY:** Cyclophosphamide **DTH:** Delayed-type hypersensitivity **LPS:** Lipopolysaccharide **ATx:** Adult thymectomy (thymectomized) **SEM:** Standard error of the mean **PFC:** Plaque-forming cell **SRBC:** Sheep red blood cells