SPECIFIC SUPPRESSOR CELLS IN MICE BEARING A SYNGENEIC MASTOCYTOMA

by

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ABSTRACT

P815 X2 mastocytoma cells, when injected subcutaneously into syngeneic DBA/2 mice, induced T lymphocyte mediated cytotoxicity in the mice. During the course of tumor growth this cytotoxic activity decreased and ultimately the tumor killed the mice. General immunological reactivity of lymphocytes from tumor bearing mice measured by their proliferative response to mitogens remained unaffected.

In order to elucidate the mechanism of the decrease in cytotoxicity in the later stages of tumor growth, an in vitro method to generate cytotoxic cells against syngeneic P815 cells was developed. When spleen cells from mice with tumors in early stages of growth were incubated in vitro with mitomycin C-treated tumor cells, specific cytotoxicity mediated by T lymphocytes was greatly enhanced. In contrast, spleen cells, taken from mice with tumors at a later stage in their growth or from normal untreated mice, did not develop cytotoxic activity. Serum from P815 tumor bearing mice did not have a direct inhibitory effect on the cytotoxicity. The unresponsiveness of spleen cells from mice with tumors in the later stages of growth seemed to be due to the presence of suppressor cells since in vitro generation of cytotoxicity by spleen cells from early tumor-bearing mice was inhibited by the addition of spleen cells or thymocytes from mice with progressively growing tumors. Normal spleen cells or thymocytes did not affect the response. Suppressive lymphoid cells from tumor bearing mice did not inhibit the mitogen responses of normal spleen cells.

The suppressor cells in P815 tumor bearing DBA/2 mice were further characterized. Suppressive activity was almost completely eliminated by treating these cells with anti θ serum and complement. Treatment with

anti mouse Ig serum and complement or with carbonyl iron did not affect their suppressive activity indicating that the suppressor cells are T lymphocytes.

It was found that cytotoxicity against L1210 leukemia line in DBA/2 mice could also be generated in vitro by the same method as described for the P815 tumor by incubating spleen cells from mice with L1210 tumors in an early stage of their growth with mitomycin C-treated L1210 cells. When suppressive thymocytes from P815 tumor bearing mice were tested for their capacity to inhibit the generation of anti-L1210 cytotoxicity, they did not affect the activity, indicating that the suppressor cells in P815 tumor bearing mice are specific to the tumor.

When ficoll-hypaque density cell separation was carried out using cytotoxic spleen cells and suppressive spleen cells from P815 tumor bearing mice, the dense fraction was enriched for killer cells while the suppressive activity was mainly recovered in the light fraction. Therefore, killer cells and suppressor cells in P815 tumor bearing mice are thought to be distinct populations although they both belong to the T lymphocyte group.

Lymphoid cells from P815 tumor bearing mice were tested for suppressive activity at various stages of tumor growth. Suppressive activity was first detected in thymuses in the early stages of tumor growth when spleens and lymph nodes had some cytotoxic activity. The suppressive activity of thymocytes persisted during the stage of slowed tumor growth when highly cytotoxic activity could be detected in the spleens and lymph nodes. After the tumors resumed accelerated growth, lymph node cells became suppressive. In the late stage of tumor growth spleen cells as well as lymph node cells and thymocytes were suppressive.

A cell-free extract, prepared by freeze-thawing suppressive thymocytes from P815 tumor bearing mice, was also suppressive and its activity was

specific, i.e. it inhibited the generation of anti-P815 cytotoxicity but not anti-L1210 cytotoxicity.

A possible role of the suppressor cells in the present study in the escape of tumors from the host's immune system and in the regulation of the cellular immune response is discussed.

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INTRODUCTION

1. IMMUNE RESPONSES AGAINST TUMORS

It is now well recognized that most tumors in experimental animals as well as humans are antigenic to the hosts. Animals can be immunized against syngeneic tumors by various methods. Tumor cells treated with mitomycin C or irradiation to inhibit their cell division, when injected into syngeneic animals, induce specific immunity in some cases (Klein et al., 1960; Revesz, 1960). Chemical modification of tumor cells surface by glutaraldehyde (Sanderson and Frost, 1974), iodoacetate (Prager et al., 1971), or neuraminidase (Currie and Bagshawe, 1969) seems to enhance the antigenicity of tumor cells, and animals that received the modified tumor cells developed immunity to the original tumors. Surgical removal of certain type of tumors also induced specific immunity (Barski and Youn, 1969). Animals immunized against tumors by these methods may reject subsequent challenge of the same tumors but not unrelated tumors implying the presence on tumor cells of specific antigens. It seems that this tumor immunity is mediated mainly by immune lymphocytes and macrophages and that humoral immunity plays little role in the rejection of tumors (Burnet, 1971). Cell mediated immune responses against tumors have been also detected in tumor bearing animals or human patients by various *in vitro* assays such as the lymphocyte proliferation assay (Vanky et al., 1971), macrophage migration inhibition (Bloom, 1971), cytotoxicity (Takasugi and Klein, 1970), and cytostasis assay (Chia and Festenstein, 1973). These assays can generally be correlated to *in vivo* measurement of tumor immunity such as infiltration of lymphoid cells into the tumor mass or tumor neutralization upon adoptive transfer (Winn, 1959).

2. FAILURE OF THE IMMUNE RESPONSE TO REJECT TUMORS

Although immune responses against tumors have been detected in the tumor bearing host without preimmunization, tumors normally grow progressively and finally kill the host. To explain this apparent paradox, a number of mechanisms by which tumors escape from the host's immune system have been proposed:

(a) Immunosurveillance

The immunosurveillance theory (Burnet, 1971) suggests that tumor cells appear in hosts very often, but most of them are killed off by the immune system. Therefore, only weakly antigenic tumors escape from the immune system and the immunity induced in hosts is simply too weak to reject rapidly growing tumors. This theory was supported by the observations that human patients with immunological deficiency diseases have a higher incidence of cancer. Similarly, immunosuppression of the recipients with kidney transplants resulted in a higher than normal incidence of cancer. However, cancer in these patients is restricted to mainly lymphoid origin.1 Recent studies on the tumor incidence in immunosuppressed mice (Simpson and Nehlsen, 1971) and nude mice (Rygaard and Polusen, 1975) which have no cellular immunity have shown that spontaneous tumors did not develop in these mice, while a higher incidence of virus induced tumors did, presumably due to their defect in anti-virus immunity (Houghton and Whitmore, 1975). These observations are sufficient to throw some doubt on the immunosurveillance theory.

(b) Immunostimulation

The immunostimulation theory by Prehn (Prehn and Lappe, 1971) suggests that the immune responses against spontaneous tumors are normally very weak and that a weak immune reaction, rather than inhibitng tumor growth, may

actually stimulate it. Faster tumor growth in the presence of very weak immune reactions has been observed in some spontaneous tumor systems, but its mechanisms are unknown. It is not yet clear if the 'immunostimulation' effect is really involved in the escape of tumors from the immune response especially in the case of virus or chemically induced tumors which normally induce stronger immune responses.

(c) Modulation

The TL antigen on mouse lymphoma cells is lost when the tumors grow in mice which have been immunized against TL antigen, but the cells regain it when transferred to non-immune mice (Old et al., 1968). Many other cell surface antigens are known to move on the surface or to disappear from it altogether under the influence of antibody. This is attributed to the two dimensional agglutination of surface antigen by antibody and subsequent gathering of antigens into patches which lead to endocytosis (Taylor et al., 1971). It was suggested that the same mechanisms might enable tumor cells to escape from the immune response to tumor cell surface antigens. However, it is well known that at least some surface antigens on tumor cells persist upon in vivo transplantations of tumor cells as well as in tissue cultures.

(d) Blocking factors

It has been reported that serum from tumor bearing animals or human patients contains factors which specifically block cytotoxicity of lymphocytes from tumor bearing hosts (Hellstrom and Hellstrom, 1974). The factors are thought to be antibodies that bind to antigenic sites on the tumor cell surface (Hellstrom and Hellstrom, 1974), or antigen-antibody complexes (Baldwin et al., 1972). It has been suggested that immune responses detected in vitro may be blocked in vivo by these factors and, therefore, are not effective to reject tumors. However, anti-tumor responses determined

in vitro, presumably in the absence of blocking factors, are progressively decreased as the tumor grows (LeFrancois et al., 1971; Whitney et al., 1974).

It has become apparent recently that none of these rather simplistic theories adequately explain the relationship of the immune response to tumor growth, and further data are being sought to clarify this obviously complex relationship.

(e) Suppression

Cell mediated immunity is often impaired in tumor bearing animals (Adler, 1971; Whitney, et al., 1974) or human patients (Krant et al., 1968; Gatti et al., 1970; Eilber et al., 1970). In vitro studies have shown that the lymphocytes of tumor bearers undergo an impaired proliferative response to mitogens or antigens (Adler et al., 1971; Whitney et al., 1974). The in vivo correlate to these observations has involved the use of skin testing. Human studies have shown that patients with progressively growing tumors frequently lack the ability to respond to recall antigens which elicit cell-mediated immunity (Eilber et al., 1970; Sugi-Foca et al., 1973). It has been reported that the serum of both cancer patients (Nimberg et al., 1975) and experimental animals bearing tumors (Whitney and Levy, 1974, 1975, a, b) contains factors which non-specifically block the response of normal lymphocytes in vitro to a number of immunological reactions including: mitogen responsiveness, mixed leukocyte reaction and specific antigen responses. However, it is not yet clear if this non-specific suppression of general immunity is the cause of the suppression of specific anti-tumor immunity or the result of progressive tumor growth caused by the suppression of specific anti-tumor immunity which involves some other mechanisms.

3. OBJECT OF PRESENT WORK

It is clear that the relationship between the immune system and tumor cells is highly complex and may involve a number of separable mechanisms. It is also clear that the original hypotheses designed to explain this relationship are no longer acceptable in the light of accumulating data in this field of study. The work reported in this thesis was undertaken to investigate status of the immune system during the progressive growth of a tumor with particular reference to recent data implicating a role for suppressor cells in the failure of the immune system to combat tumor growth.

4. SUPPRESSOR CELLS IN TUMOR BEARING HOST

Recently it has been reported that suppressor cells which inhibit various immune responses in a non-specific manner are present in tumor bearing animals. Suppressor cells in mice bearing murine sarcoma virus induced sarcomas (Kirchner et al., 1974) and methylcholanthrene induced sarcomas (Eggers and Wunderlich, 1975; Pope et al., 1976) were described as the lymphoid cells that inhibit general immune responses such as the response to mitogens (Kirchner et al., 1974) or to alloantigens in mixed leukocyte culture (Eggers and Wunderlich, 1975). Suppressor cells in mice bearing murine sarcoma virus induced sarcomas have been identified as B lymphocytes and have been shown to inhibit specific anti-tumor cytotoxicity as well as general immune responses by means of antigen-antibody complexes (Gorczynski et al., 1975). Another group of investigators found that the suppressor cells in the same system may be cells of the monocyte/macrophage series and inhibit DNA synthesis of T lymphocytes (Kirchner et al., 1975). Suppressor cells in mice bearing a methycholanthrene induced sarcoma have also been identified

as adherent cells of the macrophage/monocyte series (Pope et al., 1976).

While the foregoing are examples of generalized non-specific inhibition of immune responsiveness suppressor cells that inhibit only specific anti-tumor immune responses have also been reported, eg. in Japanese quails bearing Rous virus induced sarcomas (Hayami et al., 1972), in mice bearing sarcomas induced by Moloney sarcoma virus (Halliday,11971, 1972) or methyl-cholanthrene (Fujimoto et al., 1976, a, b) and in rats bearing mammary-adenocarcinomas (Kuperman et al., 1975). This type of suppressor cell in mice bearing sarcomas induced by Moloney sarcoma virus or methylcholanthrene were found to be specific for the given tumor studied (Halliday, 1972; Fujimoto et al., 1976 a) and belong to the T lymphocyte population (Fujimoto et al., 1976 a, b).

Although the relationship between nonspecific suppressor cells and the suppressor cells that inhibit specific anti-tumor immune responses is unknown, it is thought that both of them can inhibit specific anti-tumor immunity which may result in progressive tumor growth.

5. SUPPRESSOR T CELLS IN IMMUNE REGULATION

T lymphocytes are known to have various functions. Upon stimulation by antigens or mitogens, they produce various factors such as migration inhibition factor, etc., differentiate into cytotoxic killer cells, and cooperate with B lymphocytes as helper cells in antibody production. More recently another function of T lymphocytes -- the suppressor that inhibits immune responses -- has been recognized and thought to be involved in regulation of immune responses (Gershon, 1975). Suppressor T cells have been extensively studied in various systems involving antibody formation.

When animals were tolerized to sheep red blood cells, thymocytes from tolerant animals specifically suppressed the antibody response to the same antigen in normal syngeneic animals (Gershon and Kondo, 1971). The same effects of T lymphocytes have been confirmed in many other systems of immunological tolerance and have been reviewed extensively elsewhere (Nachtigal et al., 1975). It has been suggested that suppressor T cells are involved in self-tolerance and that a deficiency of suppressor cells may therefore contribute to the etiology of autoimmunity (Gerber et al., 1974).

Suppressor T cells were also found in rats immunized with dinitrophenylated Ascaris-extract (DNP-Asc) (Tada et al., 1975). When thymocytes or spleen cells from these rats were transferred to recipient rats which were producing high and persistent Ig E antibody against DNP-Asc, they specifically suppressed the ongoing Ig E antibody response against DNP-Asc.

Suppression of antibody allotype also seems to involve suppressor T cells. When male SJL/L mice which carry one allotype and female BALB/c mice carrying another allotype were mated, immunization of BALB/c mice with SJL/L allotype before mating completely or chronically suppressed the production of SJL/L allotype in more than half the progeny by the time they were six months old (Jacobson et al., 1972). Subsequently it was found that an active factor associated with T lymphocytes is responsible for chronic allotype suppression, because T lymphocytes from chronically suppressed progeny suppressed production of paternal allotype by unsuppressed hybrid spleen cells (Herzenberg et al., 1975).

Histocompatibility linked genetic unresponsiveness of some strains of mice to certain synthetic peptide antigens seem to be another immunological phenomenon that involves the activities of suppressor T cells. It was found

that B lymphocytes specific for the peptide failed to cooperate with T lymphocytes and that this failure was due to the presence of suppressor T cells at least in some of the non-responder mice (Benacerraf et al., 1975).

The role of suppressor T cells in the cellular immune response is not yet clear, although some in vitro studies suggest they may be involved. When splenic T lymphocytes were stimulated by Concanavalin A, the stimulated cells were found to inhibit the ability of T lymphocytes to differentiate into cytotoxic cells. (Peavy and Pierce, 1974). Similar non-specific suppressor T cells have been spontaneously generated when normal spleen cells were incubated in vitro for 3 - 4 days without antigens or mitogens (Burns et al., 1975). However, the immunological significance of these suppressor cells is unknown, and their analogues in vivo have yet to be demonstrated.

On the other hand, the regulatory role of thymocytes in the cellular immune response has been suggested by various observations in vivo. Gershon et al. (1974) reported such effects of thymocytes in graft-versus-host reactions induced by the inoculation of parental thymocytes into F_1 mice. When the spleen localizing fraction of parental thymocytes were removed by splenectomy after the inoculation of parentalccells, the response of parental cells against F_1 was either enhanced or suppressed depending on the timing of the splenectomy. This observation suggests that the effect of regulatory cells in the thymus is bidirectional.

The regulatory role of thymocytes has been also suggested in the generation of cytotoxicity against allogeneic cells. Simpson and Cantor (1975) reported that adult thymectomy increased the ability of T lymphocytes to generate primary cytotoxic responses, but had little effect on the development of cytotoxic T memory activity.

Similar effects of thymectomy were observed in autosensitization

against syngeneic cells (Carnaud et al., 1975). When mouse lymphocytes were exposed to syngeneic fibroblasts in Millipore chambers inserted into the peritoneal cavity of thymectomized mice, cytotoxic cells directed against syngeneic fibroblasts were induced. This effect was inhibited by thymic extract suggesting a possible role of suppressor T cells in the maintenance of self tolerance.

These results support the contention that suppressor T cells are also involved in the regulation of the cellular immune response. Direct evidence may in fact be provided by studies in tumor systems.

MATERIALS AND METHODS

Mice

Female DBA/2, CBA and BALB/C mice were obtained from the Jackson Laboratory (Bar Harbor, Maine).

Tumor

P815X2 mastocytoma and L1210 lymphocytic leukemia in DBA/2 mice were obtained from Dr. J.B. Smith (Institute for Cancer Research, Philadelphia, Pennsylvania). P388 lymphocytic leukemia in DBA/2 mice and S49A lymphoma in BALB/C mice were obtained from the Salk Institute for Biological Studies (San Diego, California). P815 and P388 tumors were maintained in the ascites form by serial transplantation by intraperitoneal injection of the tumor cells from ascites fluid. The S49A tumor prepared originally from a subcutaneously growing tumor in BALB/C mice and the L1210 tumor from ascites fluid in DBA/2 mice were maintained in tissue cultures using RPMI 1640 medium (Grand Island Biological Company, Grand Island, N.Y.) containing 10% heat inactivated fetal calf serum (FCS, Grand Island Biological Company) and supplemented with 100 units/ml penicillin and 10 µg/ml streptomycin. This medium was used for cell cultures throughout the present study and is designated as RPMI 1640 culture medium.

CBA mice, which differ at the H-2 locus from DBA/2 mice, rejected 10^7 P815 cells injected intraperitoneally. BALB/C mice which are H-2 identical but differ at the M locus and the minor histocompatibility antigens rejected 10^5 P815 cells injected subcutaneously. Intraperitoneal injection of 10^2 P815 cells or subcutaneous injection of 2×10^3 cells into DBA/2 mice always induced tumors which grew progressively and killed the animals.

Preparation of lymphoid cells and tumor cells

Mice were sacrificed by cervical dislocation, their spleens, thymuses or lymph nodes removed asceptically, teased apart in phosphate buffered saline (PBS) containing 5% FCS and the cell clumps disaggregated by either expulsion through tuberculin syringe with a #26 gauge needle or by passing small pieces through a 60 mesh stainless steel mesh. Red blood cells were lysed by treating spleen cells with 0.14M NH_4Cl solution in PBS for two minutes. The cells were then washed twice with PBS containing 5% FCS.

Cells from ascites tumors or tissue cultures were washed twice with PBS containing 5% FCS. Cell counts were carried out by direct microscopic examination and their viability assessed by the trypan blue exclusion method.

Cytotoxicity assay

Target cells for the cytotoxicity test were labeled with ^{51}Cr-sodium chromate (New England Nuclear, Boston, Mass.) as described by Brunner et al. (1968) with slight modifications. Two million target cells were incubated with 100-200 µCi ^{51}Cr-sodium chromate in 0.5 ml. RPMI 1640 culture medium for one hour at 37°C. The labeled cells were washed four times with PBS, resuspended in RPMI 1640 culture medium and incubated for one hour. Immediately before the assay the target cells were harvested by centrifugation, resuspended in the medium and counted. The effector cells to be tested and 10^4 labeled target cells were mixed at various ratios in either 12 X 75 mm round bottom plastic tubes (Falcon, #2003, Oxnard, California) or in the well of microculture plates (Linbro Chemical, IS-FB-96-TC, New Haven, Conn.). The final volume was adjusted to 0.4 ml. in case of plastic tubes and 0.25 ml for microculture plates. The cells were incubated at 37°C for either

6 hours or 18 hours in a humidified incubator with 5% CO_2, 95% air. After incubation the cells were sedimented by centrifugation (200 x g, 5 min.) and 0.2 ml (for plastic tubes) or 0.1 ml (for microculture plates) of the supernatant was removed for counting of ^{51}Cr on a gamma counter (Beckman Biogamma). Percent specific cytotoxicity was calculated as follows:

$$\% \text{ specific cytotoxicity} = \frac{\text{test release (cpm) -spontaneous release (cpm)}}{\text{maximum release (cpm) - spontaneous release(cpm)}} \times 100$$

Spontaneous release was measured by incubating 10^4 target cells alone, and maximum release was obtained by lysing 10^4 target cells by either freeze-thawing three times or treating with 5% triton X 100 (Sigma Chemical Company, St. Louis, Missouri). In some cases the control release measured by incubating 10^4 target cells with normal spleen cells was used in place of the spontaneous release. However, the control release was never significantly higher than the spontaneous release.

In vitro generation of cytotoxic cells

Tumor cells (5 X 10^6/ml) were incubated with 50 µg/ml. mitomycin C (Sigma Chemical Company) in RPMI 1640 culture medium at 37°C for one hour, then washed with PBS three times and resuspended in medium. Mitomycin C treated tumor cells and 10^7 lymphoid cells in RPMI 1640 culture medium supplemented with 5 X 10^{-5}M 2-mercaptoethanol were dispensed into 17 X 100 mm plastic tubes (Falcon, #2001) and the final volume was adjusted to 2.5 ml. The tubes were incubated at 37°C for various times, then the cells were harvested by centrifugation, washed and resuspended in medium, after which the viable cells were counted and tested for cytotoxicity. The mitomycin C treated tumor cells did not survive more than 48 hours and, therefore, were not apparent in the cultures at the time of harvesting cytotoxic cells.

Suppression experiments

DBA/2 mice were injected subcutaneously with a low dose of P815 cells (10^4 or 2 x 10^3) or L1210 cells (10^3). After 13-16 days for P815 tumors or 10-12 days for L1210 tumors, spleens were used as a source of immune cells. Lymphoid cells to be tested for suppressive activity from mice bearing large tumors were mixed with immune spleen cells in culture to generate cytotoxicity against P815 or L1210 cells. The suppressive activity was estimated from the decrease in the cytotoxicity as compared to control cultures in which normal lymphoid cells were added. The total number of lymphoid cells were always adjusted to 10^7. Per cent suppression was calculated from the decrease in cytotoxicity at a fixed effector/target cell ratio. When the degree of suppression had to be quantitated more precisely, cytotoxicity was tested at various effector/target cell ratios and the decrease in the total number of lytic units was assessed. One lytic unit, which was defined as the number of effector cells required to lyse 50% of 10^4 target cells in 18 h incubation was estimated by linear regression analysis of per cent cytotoxicity versus logarithm of effector/target cell ratio. Total number of lytic units were calculated from cell recovery in cultures.

Mitogen stimulation of spleen cells

Spleen cells were tested for the proliferative response to concanavalin A (Con A), phytohemagglutinin (PHA) and lipopolysaccharide (LPS) as described elsewhere (Whitney and Levy, 1974). In short, 5 x 10^5 spleen cells were cultured with 4 µg/ml Con A, 0.5% PHA or 10 µg/ml LPS in 0.25 ml RPMI 1640 culture medium (5% FCS). After 2 days for LPS stimulation or 3 days for Con A and PHA stimulation, 1.0 µCi of tritiated thymidine was added to each culture. Sixteen hours after isotope addition the cells were harvested, washed and counted with a Nuclear Chicago Unilux 1 scintillation counter.

Carbonyl Iron Treatment

5 - 10 X 10^6 spleen cells were suspended in 2.0 ml of medium and 200 mg of carbonyl iron powder (General Aniline and Film Company, New York, New York) was added. The cells were incubated for 1 hour at 37°C in 17 X 100mm plastic tubes (Falcon). The tubes were then placed on top of a magnet and the supernate was removed by pasteur pipette. This procedure was repeated three times.

Anti θ serum and Complement Treatment

Antiserum against brain associated θ antigen was raised in rabbit by injecting DBA/2 brain in complete Freund's adjuvant as described elsewhere (Kelly et al., 1974). The antiserum was extensively absorbed with DBA/2 mouse liver. Five million cells to be treated were suspended in 0.5 ml of a 1/4 dilution of anti θ serum and 0.5 ml of a 1/5 dilution of guinea pig complement. The mixture was incubated for 1 hour at 37°C, washed with PBS three times, resuspended in the medium and viable cells were counted. This treatment was cytotoxic to T lymphocytes but not to B lymphocytes as indicated by the elimination of responses to Con A and PHA without a concomitant reduction in the LPS response or numbers of plaque forming cells.

Anti mouse Ig serum and complement treatment

Antiserum against mouse Ig was raised in sheep by injecting the Ig fraction prepared from mouse serum as described elsewhere (Whitney et al., 1974). The antiserum was absorbed with DBA/2 mouse thymocytes (10^8 thymocytes for 1 ml of antiserum). Spleen cells were treated with anti Ig serum and complement in an identical manner to that described for anti θ serum and complement treatment. This treatment was cytotoxic to B lymphocytes but not to T lymphocytes as indicated by the elimination of response to LPS or plaque forming cells without concomitant reduction in Con A and PHA responses.

Nylon Wool column

Spleen cells were depleted of B lymphocytes by adherence to nylon wool as described by Julius et al. (1973). In short, 10^8 spleen cells were put onto a column containing 0.6 g nylon wool which had been washed extensively with distilled water, dried, autoclaved and washed again with PBS containing 5% FCS. The column was incubated for 45 minutes at 37°C, then the cells which did not adhere to the nylon wool were eluted from the column with PBS containing 5% FCS. This fraction of cells showed a high mitogenic response to Con A and PHA but did not respond to LPS.

Ficoll-hypaque density cell separation

Ficoll-hypaque was prepared from stock solutions of ficoll 400 (Pharmacia Fine Chemicals, Uppsala, Sweden) and sodium hypaque (Winthrop Laboratories, Aurora, Ontario) by the method described elsewhere (Pope et al., 1976). Spleen cells were suspended at a concentration of 4.0×10^7 cells per ml in 2.5 ml of PBS. This suspension was carefully layered onto 2.5 ml of ficoll-hypaque (density 1.06) in a 17 x 100 mm plastic tube (Falcon, #2001). The tube was centrifuged for 30 minutes at 400 x g. The cells found in the supernate were collected with a pasteur pipette, washed with PBS and resuspended in medium. This fraction was designated as the light fraction ($d < 1.06$). The cells sedimenting to the bottom of the tube were washed and resuspended in PBS, then further separated by repeating the same procedure as described above using ficoll-hypaque with a higher density (density 1.08). The cells in the supernatant fraction, designated as the medium fraction (density between 1.06 and 1.08), and those sedimenting to the bottom of the tube, designated as dense fraction ($d > 1.08$), were washed with PBS, resuspended in medium and viable cells were counted by trypan blue exclusion.

In some experiments spleen cells were separated in one step using ficoll-hypaque with a density of 1.08 only. This gives two fractions, i.e. the dense ($d > 1.08$) and the light ($d \leq 1.08$) fractions.

Preparation of thymocyte extracts

A single cell suspension was prepared from the thymuses of normal or P815 tumor bearing DBA/2 mice. The cell suspension at the concentration of 5 X 10^7/ml in PBS was freeze-thawed three times, centrifuged for 20 minutes at 400 x g and the supernatant was collected. This cell free extract was sterilized by filtration through millipore membrane (pore size 0.2 μ) before use.

RESULTS

Growth of Tumor and Cytotoxicity of Spleen Cells

DBA/2 mice were injected subcutaneously with 5×10^4 syngeneic P815 mastocytoma cells. At various times following injection of tumor cells, the mice were sacrificed, tumor weight was measured and spleen cells were tested for cytotoxicity against tumor cells and their proliferative response to Con A, PHA, and LPS was assessed. Solid tumors were first observed on day 8 and the tumor continued growing until day 12. After day 12 tumor growth slowed. Specific cytotoxicity of the tumor-bearer spleen cells increased markedly during this period of slowed tumor growth (days 12-16). Figure 1 represents the average of data obtained from 6 animals for each data point. Tumor growth in living animals was measured with calipers, and during this period of high cytotoxicity, tumor growth frequently stopped completely and often showed some regression. However at about day 16 to 18, specific cytotoxicity dropped, and this coincided with accelerated tumor growth. The mice were usually killed by the tumor 20 - 28 days after injection. The cytotoxicity detected in this system appears to be mediated by T lymphocytes, because it was abolished by anti θ and complement treatment but was not affected by nyloon wool column separation (Table I).

If a lower dose of tumor cells (10^4) was injected, the phase during which tumor growth slowed and regressed was accentuated. In these animals, also, there was a peak of specific cytotoxicity followed by a decline. As with those animals receiving 5×10^4 cells, tumor growth eventually accelerated and killed the animals. A typical set of data are presented in figure 2.

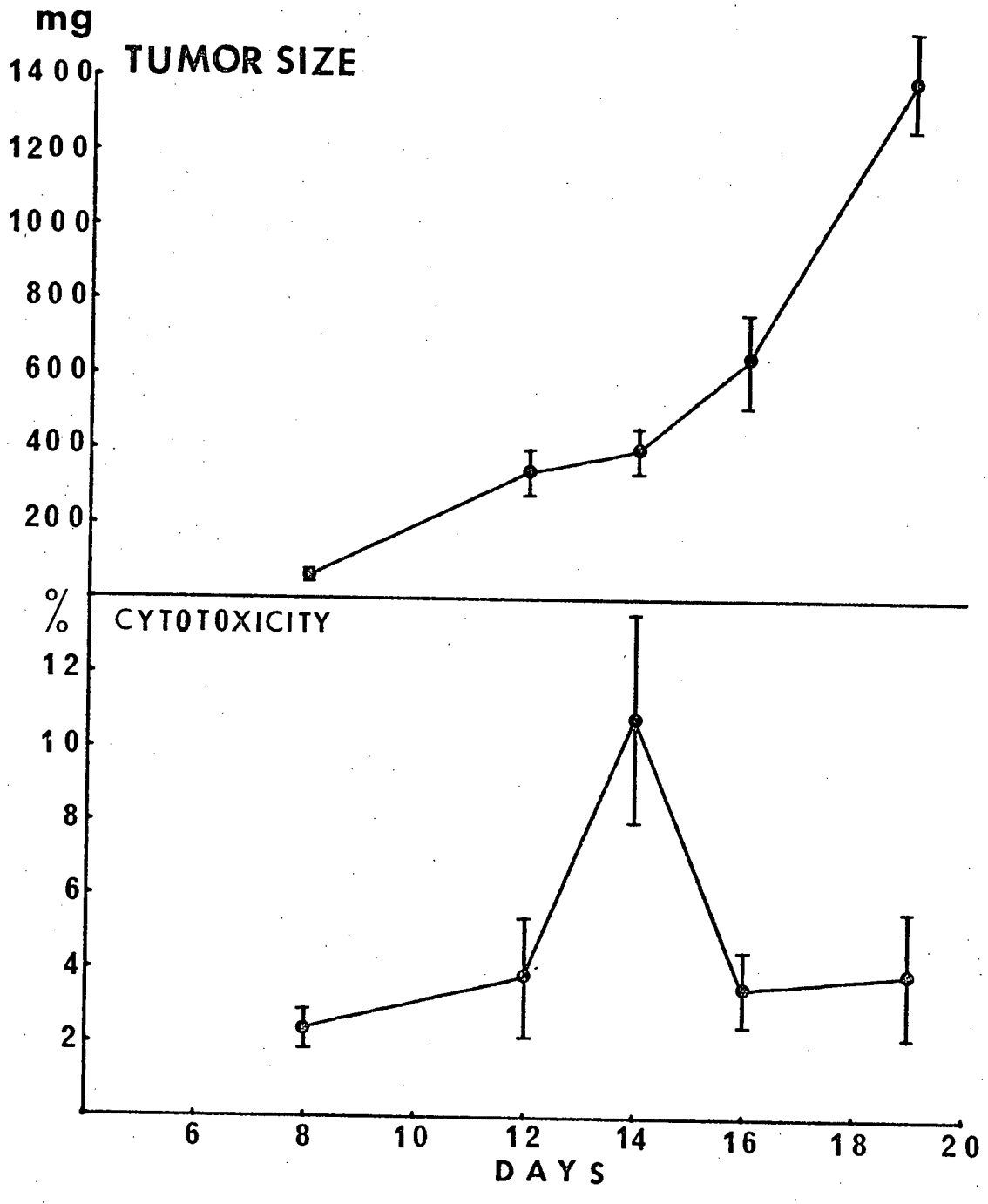

Figure 1. Growth of P815 tumor and cytotoxicity of spleen cells from P815 tumor-bearing mice. The mice received 5 X 10^4 P815 cells subcutaneously. Tumor size was measured by the weight of the excised tumors. The cytotoxicity of spleen cells were tested by ^{51}Cr release assay. The spleen cell to target cell ratio was 200:1 and the cells were incubated for 18 hr. Each data point represents the average of data obtained from 6 animals and vertical bars show standard error of the mean.

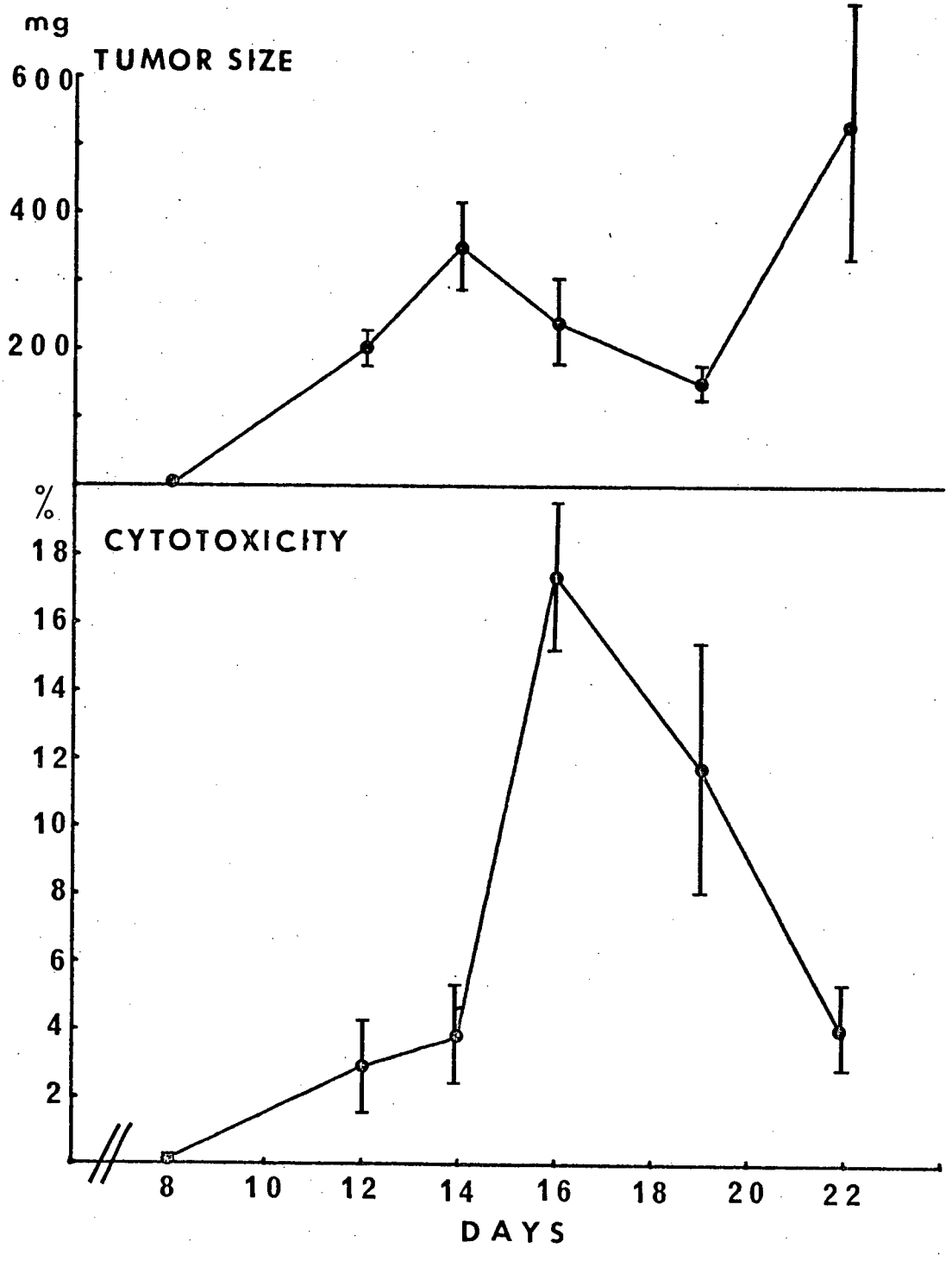

Figure 2. Growth of P815 tumor correlated with cytotoxicity of spleen cells from tumor-bearing mice. The mice received 10^4 P815 cells subcutaneously. Tumor size was measured by weight of the excised tumors. The cytotoxicity of spleen cells were tested by ^{51}Cr release assay. The spleen cell to target cell ratio was 200:1 and the cells were incubated for 18 hr. Each data point represents the average of data obtained from 5 animals and bars show standard error of the mean.

TABLE I. CHARACTERIZATION OF CYTOTOXIC CELLS IN SPLEENS FROM P815 TUMOR BEARING MICE

	Treatment of cells (a)	Specific cytotoxicity ±SEM (b) (%)
Exp. I.	none	10.5 ± 0.3
	anti θ + C'	0.2 ± 0.2
Exp. II.	none	6.6 ± 0.4
	nylon wool column	17.6 ± 0.9

(a) Spleen cells from mice bearing small P815 tumors (14 days after subcutaneous injection of 5 X 10^4 P815 cells) were used as a source of cytotoxic cells.

(b) Cytotoxicity against P815 cells was tested by ^{51}Cr release assay. The spleen cell to target cell ratio was 200:1 and the cells were incubated for 18 hr. The figures are the means of triplicate tests.

The proliferative response of spleen cells to Con A, PHA and LPS did not change significantly up to day 19 (Table II). After day 19 the tumor often metastasized to spleen or peritoneal cavity and the cytotoxicity and the mitogenic responses could not be properly tested because of the high backgrounds due to the tumor cells.

In vitro Generation of Cytotoxicity

In an attempt to elucidate the cause of the decrease in cytotoxicity which was observed after 14 days of tumor growth an in vitro method to generate cytotoxic cells against syngeneic mastocytoma cells was developed. When spleen cells from mice with small tumors (10-12 days after injection of tumor cells) were incubated in vitro with mitomycin C treated P815 cells, significant levels of cytotoxicity could be demonstrated. The activity was dependent on the incubation time and the dose of mitomycin C treated tumor cells; the highest cytotoxicity being obtained with 10^7 spleen cells were incubated with 5×10^5 mitomycin C treated tumor cells for 4 days (Fig. 3,4). Significant levels of cytotoxicity could be detected after 6 hours of incubation although in most cases we used 18 hours incubation for the ^{51}Cr release assay. Under the conditions used in these experiments, normal spleen cells did not develop significant cytotoxicity. Under the conditions described here, no intact tumor cells remained in the culture after 4 days incubation; therefore no measures needed to be taken to remove them prior to the mixing of viable spleen cells with ^{51}Cr labeled tumor cells.

To establish which cells were the cytotoxic effectors cultured spleen cells were treated in various ways, prior to testing. Cytotoxicity was completely abolished by anti θ and complement treatment, while neither B

TABLE II. PROLIFERATIVE RESPONSE OF SPLEEN CELLS FROM P815 TUMOR BEARING MICE TO MITOGENS

Days after tumor cell injection (a)	Stimulation (b) (cpm ± SEM)		
	LPS (10 µg/ml)	Con A (4 µg/ml)	PHA ((5%)
)0	47467 ± 5425	190467 ± 25881	64537 ± 7311
8	47962 ± 2691	139204 ± 7004	46455 ± 1219
12	59987 ± 3491	241806 ± 15726	67594 ± 11125
14	60516 ± 3369	233127 ± 20820	79658 ± 5385
16	51439 ± 8540	159188 ± 26737	47262 ± 7875
19	41970 ± 3565	175236 ± 26769	56982 ± 8755

(a) DBA/2 mice were injected subcutaneously with 5 X 10^4 P815 cells

(b) Mitogen stimulation was calculated from the mean radioactivity of triplicate test cultures with mitogens minus that of control cultures without mitogens. The figures are the average of data obtained from 6 animals in each group.

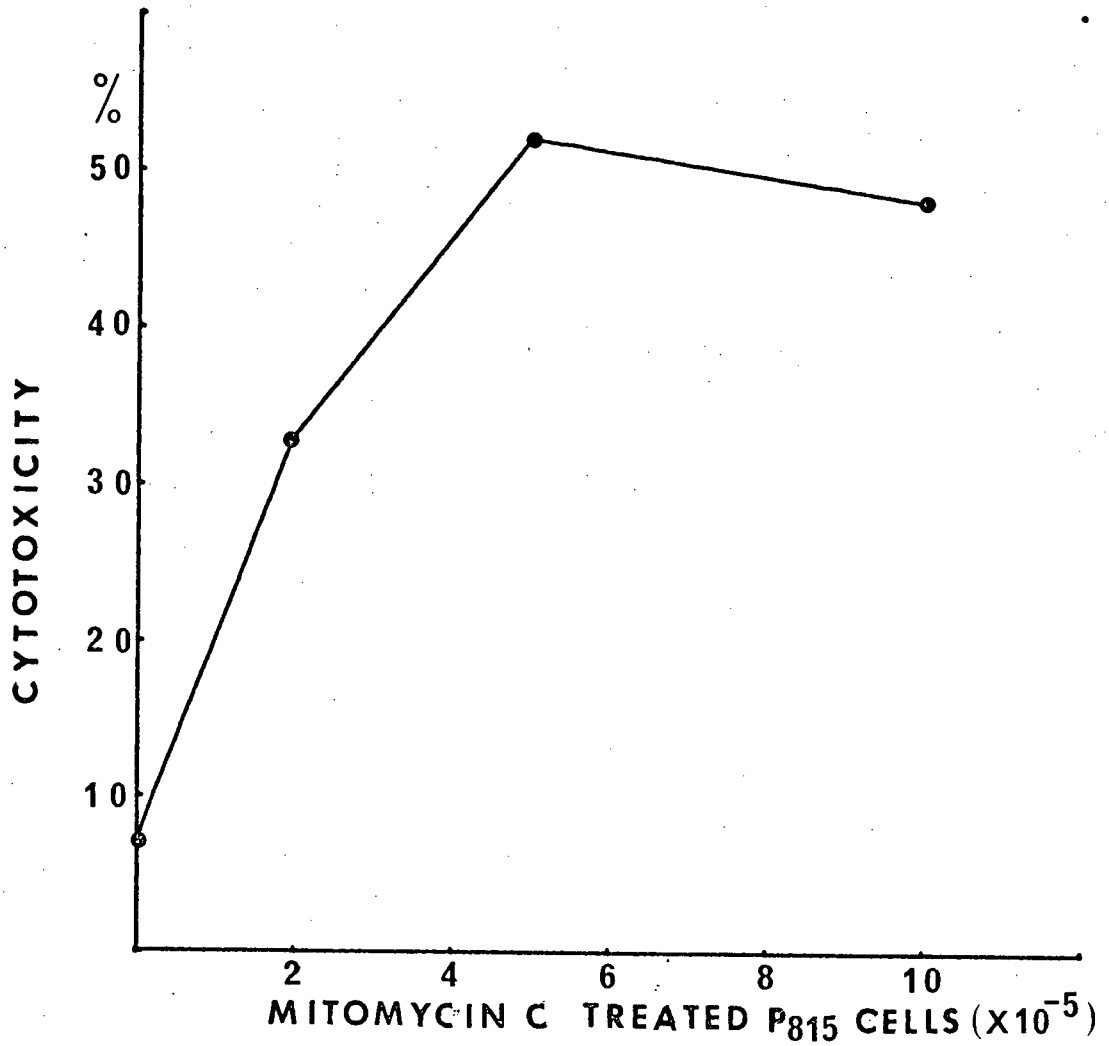

Figure 3. Effect of dose of mitomycin C-treated tumor cells on in vitro induction of cytotoxicity. Spleen cells from mice bearing small tumors (10 days after subcutaneous injection of 5 X 10^4 P815 cells) were incubated with various numbers of mitomycin C-treated P815 cells for 4 days, then tested for the cytotoxicity against P815 cells by ^{51}Cr release assay. The effector cell to target cell ratio was 50:1 and the cells were incubated for 18 hr.

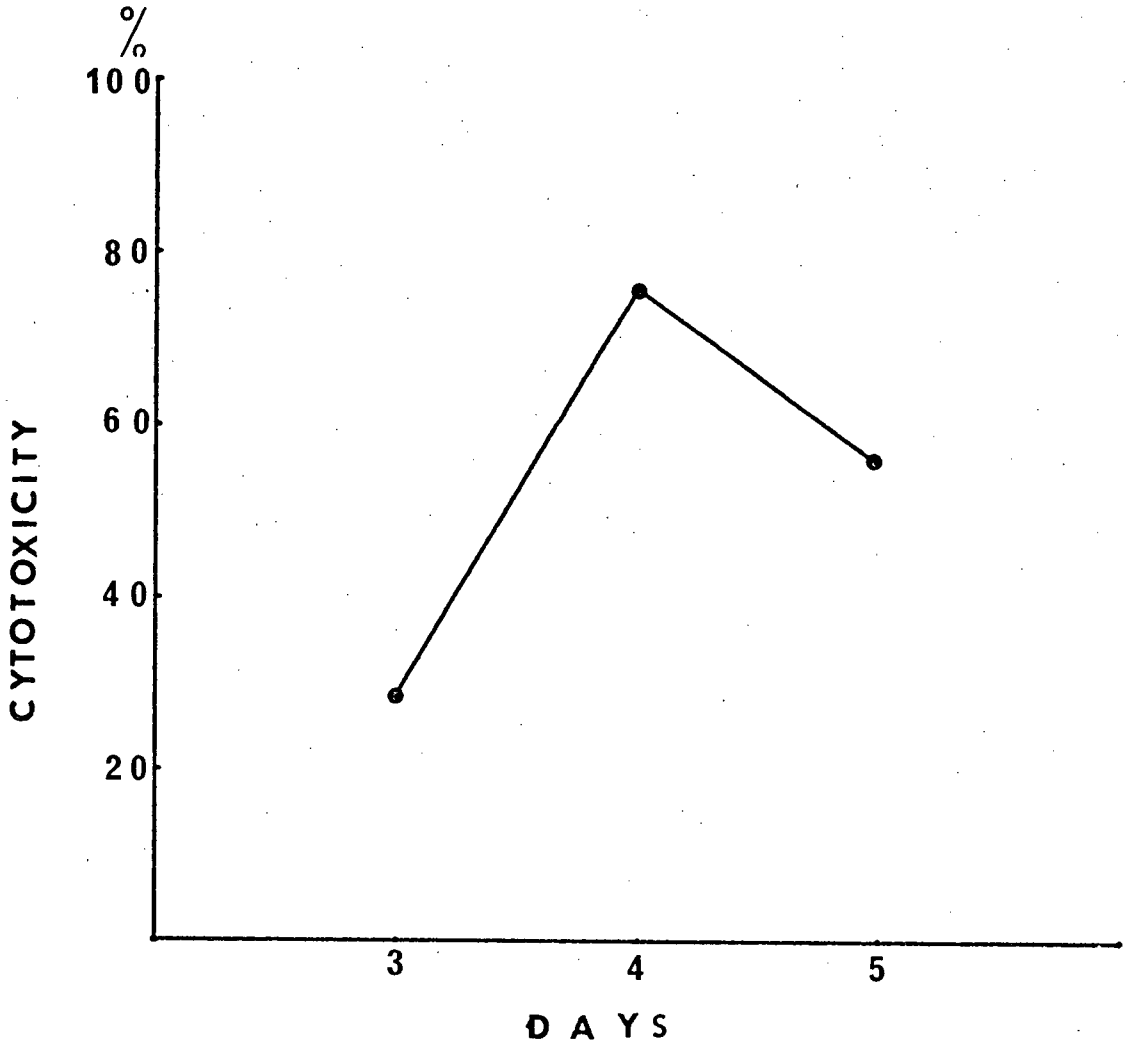

Figure 4. Incubation period for in vitro generation of anti-P815 cytotoxicity. Spleen cells from mice bearing small P815 tumors (10 days after subcutaneous injection of 5 X 10^4 P815 cells) were incubated with 5 X 10^5 mitomycin C treated P815 cells for various periods of time, then tested for anti-P815 cytotoxicity by ^{51}Cr release assay. The effector cell to target cell ratio was 25:1 and the cells were incubated for 18 hr.

TABLE III. EFFECTOR CELLS MEDIATING IN VITRO GENERATED CYTOTOXICITY

	% Cytotoxicity [a]		
	Exp 1 [b]	Exp 2 [c]	Exp 3 [c]
non-treated	12.3	35.1	36.8
anti θ + C'	0	0.2	0
carbonyl iron	10.0	38.1	34.2
nylon wool column	11.1	36.3	31.2

(a) Cytotoxicity was induced in vitro using spleen cells from small tumor bearing mice (10 days after subcutaneous injection of 5 X 10^4 P815 cells). Effector cell to target cell ratio in ^{51}Cr release assay was 50:1.

(b) Incubation time with target cells was 6 hr.

(c) Incubation time with target cells was 18 hr.

lymphocyte depletion by adherence to nylon wool nor removal of phagocytic cells by carbonyl iron treatment affected the cytotoxicity (Table III). Therefore it was concluded that the cytotoxicity was mediated by T lymphocytes. In vitro incubated cells which were highly cytotoxic to P815 cells did not show any cytotoxicity to unrelated P388 and S49A tumor cells. When normal DBA/2 spleen cells were sensitized in vitro against CBA spleen cells by the same culture method, the sensitized cells, which were cytotoxic to PHA stimulated CBA spleen cells, did not show significant cytotoxicity against P815 cells (Table IV). Therefore, the activity appears to be specific.

Effect of serum on cytotoxicity

Since it has been reported that serum from tumor bearing hosts sometimes contains 'blocking factor' which specifically inhibits anti-tumor cytotoxicity (Hellstrom and Hellstrom, 1974) or factors which nonspecifically inhibit general lymphocyte activities (Whitney and Levy, 1974), serum from DBA/2 mice bearing progressively growing P815 tumors (17 - 19 days after subcutaneous injection of 5×10^4 P815 cells) was tested for the inhibition of anti-P815 cytotoxicity.

Spleen cells from mice bearing small P815 tumors (16 days after subcutaneous injection of 10^4 P815 cells) were tested for anti-P815 cytotoxicity in the presence of serum from normal DBA/2 mice or P815 tumor bearing mice (Table V). The serum was also tested for the inhibition of the in vitro generation of anti-P815 cytotoxicity at various concentrations (Table VI).

In both experiments the serum from P815 tumor bearing DBA/2 mice, as compared to normal DBA/2 mice serum, had no inhibitory effect. Therefore, it is unlikely that the decline of cytotoxicity observed as the tumor progressed is due to the presence of 'blocking factor' or non-specific inhibitory factors in the serum.

TABLE IV. SPECIFICITY OF CYTOTOXICITY GENERATED IN VITRO

In Vitro culture		Cytotoxicity Test [a]	
Responding Cells	Stimulating cells	Target cells	% cytotoxicity
P815 bearing spleen	P815	P815	19.5***[c]
P815 bearing spleen	P815	P388	0.3[NS] [d]
P815 bearing spleen	P815	S49A	0.4[NS]
normal DBA spleen	CBA spleen	P815	1.0[NS]
normal DBA spleen	CBA spleen	CBA spleen[b]	25.5***

(a) ^{51}Cr release after 6 hr incubation, effector to target ratio 50:1.

(b) CBA spleen cells were incubated for 3 days with 1% PHA before labeling with ^{51}Cr.

(c) Statistically significant $P < 0.005$.

(d) Statistically not significant

TABLE V. EFFECT OF SERUM ON ANTI-P815 CYTOTOXICITY

Serum	concentration	Specific cytotoxicity[(a)] ± SEM
-	-	11.3 ± 1.5
normal serum[(b)]	5%	3.4 ± 0.6
tumor serum[(c)]	5%	6.2 ± 0.3

(a) ^{51}Cr release assay: effector cell to target cell ratio was 200:1, incubation period was 18 hr. The figures are the means of triplicate tests.

(b) Serum from normal DBA/2 mice.

(c) Serum from DBA/2 mice with big P815 tumors (16-19 days after injection subcutaneously with 5 X 10^4 P815 cells).

TABLE VI. EFFECT OF SERUM ON IN VITRO GENERATION OF ANTI-P815 CYTOTOXICITY

Serum	concentration	Specific cytotoxicity[(a)] ± SEM
none	-	42.6 ± 0.7
normal serum[(b)]	4%	5.6 ± 0.7
	2%	7.2 ± 0.8
	1%	10.9 ± 0.9
tumor serum[(c)]	4%	4.5 ± 0.4
	2%	4.6 ± 0.8
	1%	17.4 ± 1.1

(a) Anti-P815 cytotoxicity was generated in vitro using spleen cells from mice bearing small P815 tumors (14 days after subcutaneous injection of 10^4 P815 cells),

^{51}Cr release assay: Effector cell to target cell ratio was 50:1, incubation period was 18 hr. The figures are the means of triplicate tests.

(b) Serum from normal DBA/2 mice

(c) Serum from big P815 tumor bearing mice (16-19 days after subcutaneous injection of 5 X 10^4 P815 cells).

Suppressor Cells in Mice with Progressively Growing Tumor

In contrast to spleen cells from small tumor bearing mice, spleen cells from mice with progressively growing tumors (16 - 19 days after injection of tumor cells) did not develop cytotoxicity after incubation with mitomycin C treated tumor cells. Moreover, addition of these spleen cells to cultures of spleen cells from mice bearing small tumors inhibited the development of cytotoxicity (Table VII). This was not due simply to the dilution of reactive with unreactive cells, because the addition of normal spleen cells, which were also unreactive in culture did not diminish the response as much as did cells from progressors. The suppressive activity was also found in thymus from the same mice. However, the same suppressive spleen cells or thymocytes showed normal proliferative responses to Con A and PHA and did not affect the response of normal spleen cells to mitogens when mixed together (Table VIII). It seems unlikely that the suppression was due to metastatic tumor cells in spleen or thymus. Cells to be tested were carefully checked for the presence of tumor cells by microscopic examination before and after culture. No tumor cells were found in spleens or thymuses used in the suppression experiments, while as few as 200 tumor cells (untreated with mitomycin C) added to the incubation mixture proliferated and were easily differentiated under the microscope after the incubation. Low background counts in mitogenic response tests also support the conclusion that tumor cells were not present in spleens and thymuses used in these tests because spleen cells had very high background counts if they were contaminated with tumor cells. Therefore it seems most likely that the unresponsiveness of spleen cells from mice with progressively growing P815 tumors was due to the presence of suppressor

TABLE VII. SUPPRESSION OF IN VITRO GENERATION OF CYTOTOXICITY

Cells cultured	% Cytotoxicity[a]			
	Exp 1[b]	Exp 2[b]	Exp[c]	Exp 4[c]
normal spleen	0	1.2	0.6	0
normal thymus	0	0	0	-
late P815 spleen[d]	0	0	10.1	0
late P815 thymus	0	0	0	-
early P815 spleen[e]	22.8	11.6	46.8	89.9
early P815 spleen[f] + normal spleen	17.4	8.8	21.5	84.7
early P815 spleen + late P815 spleen	6.2	0.7	13.4	48.9
early P815 spleen + normal thymus	22.7	10.2	31.7	84.2
early P815 spleen + late P815 thymus	3.5	5.4	4.1	74.7

(a) Effector to target ratio was 50:1

(b) 6 hour incubation for ^{51}Cr release assay

(c) Overnight incubation for ^{51}Cr release assay

(d) Spleen or thymus cells from mice 16-19 days after subcutaneous injection of 5×10^4 P815 cells

(e) Spleen cells from mice 10 days after subcutaneous injection of 5×10^4 P815 cells

(f) For the mixing experiments 5×10^6 each cell population was mixed

TABLE VIII. LACK OF SUPPRESSION IN PROLIFERATIVE RESPONSE OF TUMOR BEARING SPLEEN CELLS AGAINST MITOGENS

Cells	CPM ± SEM		
	Control	Con A	PHA
normal spleen	18975 ± 442	170758 ± 3057	91415 ± 4416
P815 spleen[a]	23881 ± 965	163218 ± 4926	83374 ± 4516
normal thymus	207 ± 23	4164 ± 381	656 ± 209
P815 thymus	241 ± 22	7755 ± 491	641 ± 228
normal spleen[b] + P815 spleen	23507 ± 1964	163392 ± 6160	80749 ± 4038
normal spleen[b] + normal thymus	6012 ± 602	89898 ± 3148	37084 ± 768
normal spleen[b] ++ P815 thymus	5799 ± 315	104923 ± 6358	38045 ± 572

(a) Spleen cells from mice bearing progressively growing P815 tumors (16-19 days after subcutaneous injection of 5×10^4 P815 cells)

(b) 2.5×10^5 each cell population was mixed

(c) The figures are the mean radioactivities of triplicate test cultures.

cells which inhibited in vitro generation of cytotoxicity against P815 cells.

Preliminary studies have indicated that suppressor cells of this type may develop even in those animals in whom tumors have been resected between days 10-14 after implantation. Such animals were sacrificed at varying time intervals after tumor resection, and their spleen cells tested for cytotoxicity. At no time after resection were appreciable levels of cytotoxicity detected, and all animals so treated developed metastatic disease after a short time.

Quantitative analysis of suppression

Suppression of the in vitro generation of cytotoxicity against P815 tumor cells by the addition of spleen cells from DBA/2 mice bearing progressively growing P815 tumors was quantitatively analyzed. When the per cent cytotoxicity was plotted against the logarithm of effector/target cell ratios, a linear relationship was observed if the cytotoxicity was higher than 20% (Fig. 5). One lytic unit, which was defined as the number of effector cells to cause 50% lysis of 10^4 target cells in 18 hours incubation, was calculated by linear regression analysis of the cytotoxicity versus the logarithm of cell ratio. The total lytic units in test cultures and control cultures were obtained from cell recovery in the cultures. Then the degree of suppression was quantitated by the decrease in total lytic units in test cultures as compared to controls. No signigicant difference in cell recovery between test cultures and controls were normally observed.

Using this method the dose response of suppressor cells in the cultures to generate anti-P815 cytotoxicity was tested (Fig. 6). The degree of suppression was dependent on the dose of suppressive lymphoid cells and a higher dose of suppressive spleen cells or thymocytes caused higher suppression. In the following suppression experiments 5×10^6 suppressive spleen or thymus cells were mixed with 5×10^6 immune spleen cells.

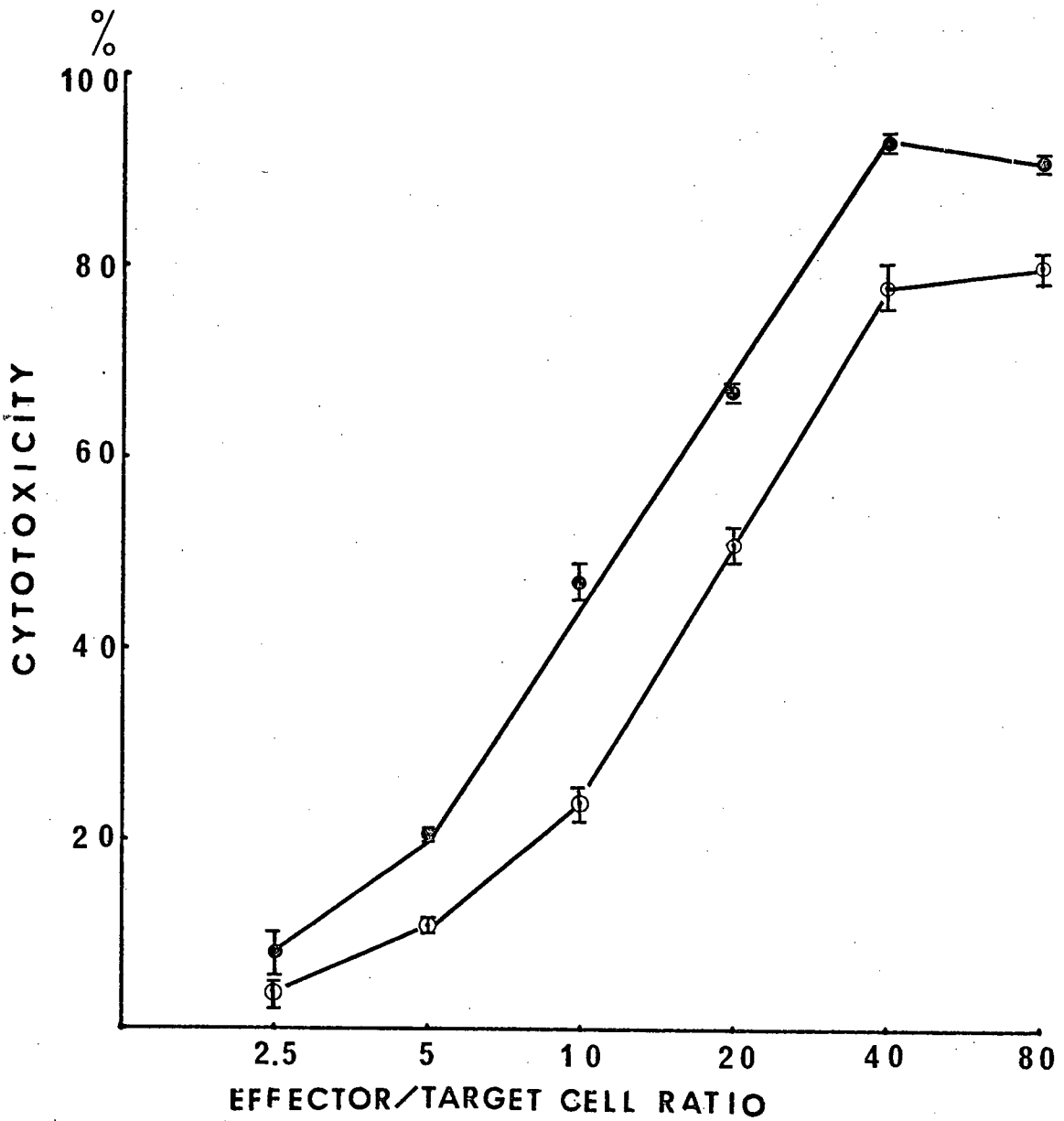

Figure 5. Linear regression analysis of the suppression of anti-P815 cytotoxicity.

Spleen cells from DBA/2 mice with small P815 tumors (14 days after subcutaneous injection of 2 X 10^3 P815 cells) were used as immune cells to generate anti-P815 cytotoxicity in in vitro cultures. Normal spleen cells or P815 tumor bearing spleen cells (19 days after subcutaneous injection of 5 X 10^4 P815 cells) were mixed with an equal number of immune cells and the total lymphoid cell number adjusted to 10^7 in each culture. The cytotoxicity test was carried out at various effector/target cell ratios and per cent cytotoxicity was plotted against logarithm of the ratio. Closed circles represent the results of control cultures containing normal spleen cells and open circles represent the results of test cultures containing tumor bearing spleen cells. Per cent cytotoxicity is the mean of triplicate tests. Vertical bars show standard error of the mean.

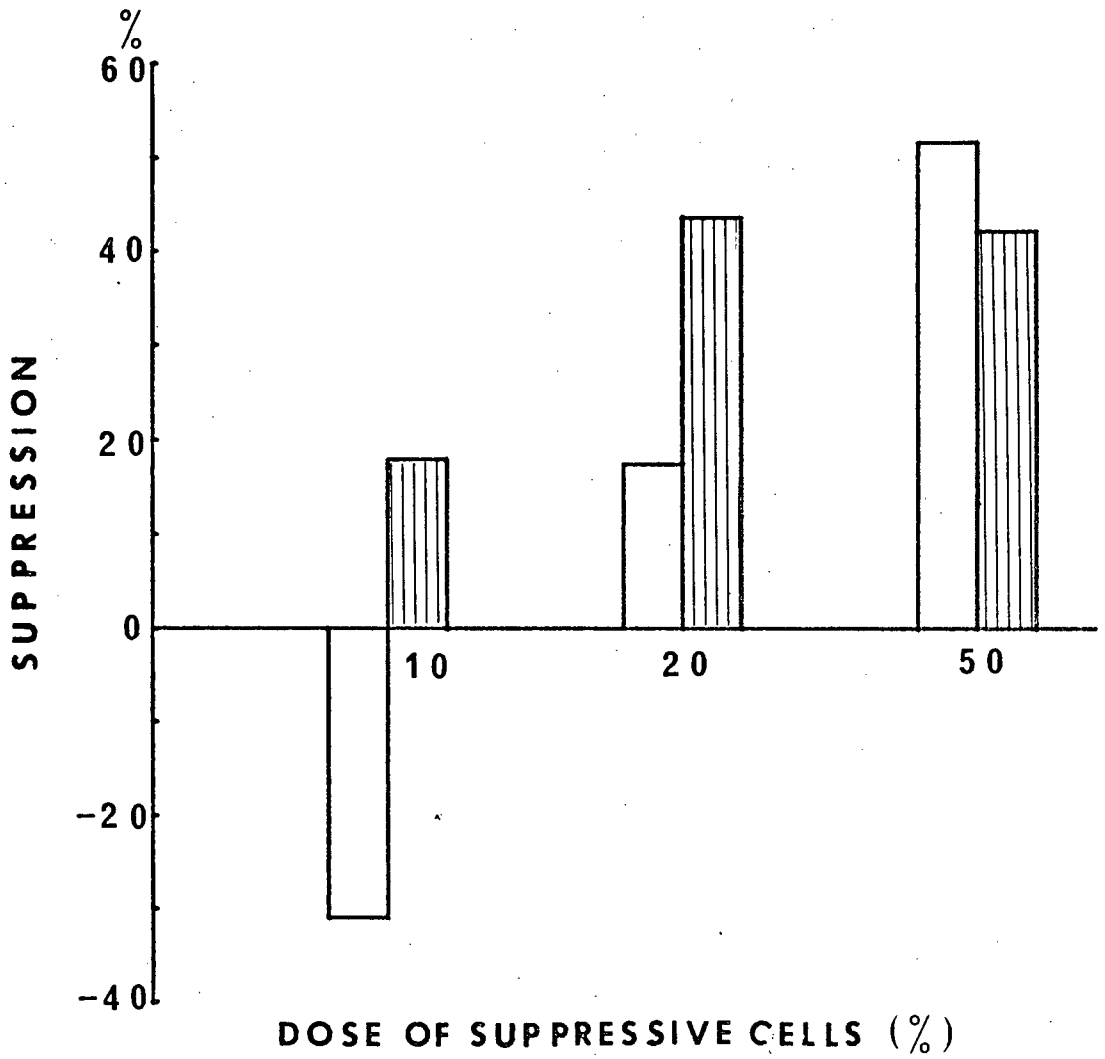

Figure 6. Effect of dose of suppressive lymphoid cells on the degree of suppression.

Spleen cells (open bars) or thymocytes (hatched bars) from mice with progressively growing P815 tumors (18 days after subcutaneous injection of 5 X 10^4 P815 cells) were used for the suppression of the anti-P815 cytotoxicity. Total lymphoid cell number was always adjusted to 10^7. Per cent suppression was estimated from the decrease in total lytic unit of the test cultures as compared to that of control cultures containing normal spleen cells or thymocytes.

Characterization of suppressor cells

Since suppressor cells were demonstrated intthymuses as well as spleens from P815 tumor bearing mice (16 - 19 days after subcutaneous injection of 5 X 10^4 P815 cells), it was considered likely that they were T lymphocytes. In order to confirm this, suppressive spleen cells were treated in various ways following which their suppressive activity was tested (Fig. 7). The activity was almost eliminated by anti θ serum and complement treatment. B lymphocyte depletion by anti mouse Ig serum and complement, or removal of phagocytic cells by carbonyl iron/magnet treatment did not affect the suppressive activity. It was concluded that the suppressor cells in P815 tumor bearing mice were T lymphocytes.

Specificity of suppressor cells

In order to determine the specificity of the suppressor cells from P815 tumor bearing mice, their effect on the generation cytotoxicity against another syngeneic tumor in DBA/2 mice was tested. DBA/2 mice were injected subcutaneously with 10^3 syngeneic L1210 leukemia cells. When spleen cells from these mice, taken 10 - 12 days after tumor cell injection, were incubated with mitomycin C treated L1210 cells in the same way as in the P815 tumor system, strong cytotoxicity against the L1210 tumor cells developed. When thymocytes from P815 bearing mice, which suppressed the generation of anti-P815 cytotoxicity, were added to the spleen cells from L1210 leukemia bearing mice, no suppression of anti-L1210 cytotoxicity was observed (Table IX). This observation supports the contention that the suppressor cells observed in this system are specific for cells exhibiting specific cytotoxicity for the P815 mastocytoma.

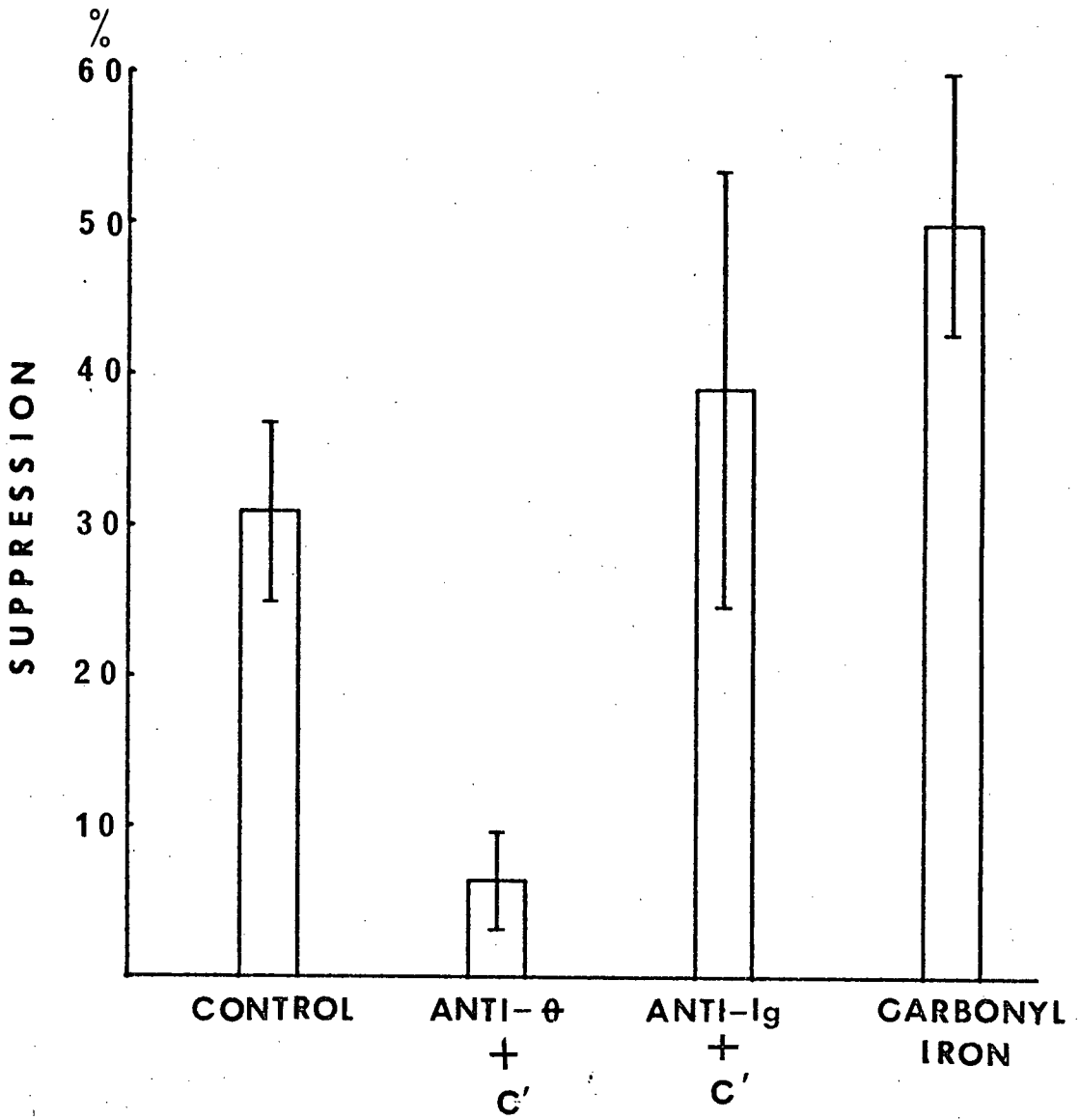

Figure 7. Characterization of suppressor cells in spleen from P815 tumor bearing mice.

Spleen cells from mice with progressively growing P815 tumors (17-19 days after subcutaneous injection of 5 X 10^4 P815 cells) were tested for the suppressive activity after various treatments. Non-treated (control) or treated cells were added to the in vitro cultures to generate anti-P815 cytotoxicity to consist 50% of the lymphoid cells in the cultures. Per cent suppression was estimated from the decrease in cytotoxicity of the test cultures at a fixed effector/target cell ratio as compared to that of the cultures containing normal spleen cells treated in identical manners. Results are the average of three experiments and vertical bars show standard error of the mean.

TABLE IX. SPECIFICITY OF SUPPRESSOR CELLS

Target	Cells cultured	% Specific Cytotoxicity[a] ± SEM Exp. 1	Exp. 2	Exp. 3	Exp. 4
P815	P815 immune[b] (10^7)	66.1 ± 2.9	52.4 ± 1.5	67.0 ± 2.0	31.6 ± 0.5
	P815 immune ($5X10^6$) + normal thymocytes ($5X10^6$)	62.9 ± 1.0	49.8 ± 2.6	31.3 ± 0.8	24.7 ± 0.3
	P815 immune ($5X10^6$) + P815 thymocytes[c] ($5X10^6$)	54.1 ± 0.5	34.7 ± 1.5	25.4 ± 2.6	18.2 ± 1.6
L1210	L1210 immune[d] (10^7)	15.8 ± 1.5	44.4 ± 1.0	36.5 ± 3.9	77.7 ± 2.8
	L1210 immune ($5X10^6$) + normal thymocytes ($5X10^6$)	14.4 ± 1.8	22.9 ± 0.4	31.2 ± 1.9	67.1 ± 1.5
	L1210 immune ($5X10^6$) + P815 thymocytes ($5X10^6$)	15.8 ± 0.9	26.1 ± 0.5	33.6 ± 4.0	79.9 ± 1.5

(a) ^{51}Cr release assay; effector cell to target cell ratio was 20:1, incubation period was 18 hours. Spontaneous release of P815 cells was 16-20%, L1210 cells 13-17%. The figures are the means of triplicate tests.

(b) Spleen cells from DBA/2 mice with small P815 tumors (14 days after subcutaneous injection of 10^4 P815 cells).

(c) Thymocytes from DBA/2 mice with progressively growing P815 tumors (16-19 days after subcutaneous injection of $5X10^4$ P815 cells).

(d) Spleen cells from DBA/2 mice with small L1210 tumors (10-12 days after subcutaneous injection of 10^3 L1210 cells).

Ficoll-hypaque density cell separation

Since both cytotoxic killer cells and suppressor cells in P815 tumor bearing mice appeared to belong to the T lymphocyte population, attempts were made to physiologically differentiate one population from the other. For this purpose ficoll-hypaque density cell separation was carried out using spleen cells from P815 tumor bearing mice, following which each fraction was tested for cytotoxicity against P815 cells or suppressive activity. When spleen cells from mice bearing small P815 tumors (14 days after subcutaneous injection of 5×10^4 P815 cells) were used, the cytotoxic cells were enriched in the dense fraction (Table X), while the suppressor activity in spleen cells from big P815 tumor bearing mice (18 - 19 days after subcutaneous injection of 5×10^4 P815 cells) was mainly recovered in the light fraction (Table XI). These results suggest that the killer cells and the suppressor cells in this tumor system are not physiologically identical. However, it may be possible that the suppressor cells in big tumor bearing mice result from a further differentiation step of killer cells.

Relationship between tumor growth and suppressive activity in lymphoid organs from tumor bearing mice

Since suppressive activity was detected in thymuses as well as in spleens from mice bearing big P815 tumors, further experiments were carried out to investigate the suppressive activities in spleens, thymuses and lymph nodes at various stages of tumor growth. DBA/2 mice were injected subcutaneously with 5×10^4 P815 cells. At various times following injection of tumor cells, three mice in each group were sacrificed, their spleen cells, thymocytes and lymph node cells were pooled separately and tested for suppressive activity (Table XII).

Suppressive activity was first detected in the thymus 8 days after

TABLE X. FICOLL-HYPAQUE DENSITY CELL SEPARATION OF KILLER CELLS

	Cell fraction[a] (cell density)	Cell recovery(%)	Cytotoxicity[b] (%) ± SEM
Exp 1	unseparated[c]	-	17.0 ± 1.0
	light ($d < 1.06$)	15.4	14.5 ± 0.8
	medium ($1.06 < d < 1.08$)	17.4	10.3 ± 1.2
	dense ($1.08 < d$)	67.2	19.0 ± 0.7
Exp 2	unseparated[c]	-	6.8 ± 2.1
	light ($d < 1.06$)	22.2	3.6 ± 1.2
	medium ($1.06 < d < 1.08$)	13.0	1.4 ± 0.8
	dense ($1.08 < d$)	64.9	13.2 ± 1.0

(a) Ficoll-hypaque density cell separation was carried out in two steps using ficoll-hypaque with density of 1.06 and 1.08.

(b) ^{51}Cr release assay: effector to target cell ratio was 200:1, incubation period was 18 hours. The results are average of quadruplicate incubation mixtures.

(c) Spleen cells from mice with small P815 tumors (14-16 days after subcutaneous injection of $2X10^3$ P815 cells) were used as a source of killer cells.

TABLE XI. FICOLL-HYPAQUE DENSITY CELL SEPARATION OF SUPPRESSOR CELLS

	Cell Fraction (density)	Suppression[c] (%)
Exp. 1[a]	unseparated[d]	17.2
	light ($d < 1.08$)	58.4
	dense ($d > 1.08$)	-46.3
Exp. 2[b]	unseparated[d]	40.6
	light ($d < 1.06$)	57.4
	medium ($1.06 < d < 1.08$)	25.5
	dense ($1.08 < d$)	34.6

(a) Cell separation was carried out in one step using ficoll-hypaque with density of 1.08.

(b) Cell separation was carried out in two steps using ficoll-hypaque with density of 1.06 and 1.08.

(c) Per cent suppression was estimated from the decrease in total lytic units of the test cultures as compared to those of control cultures containing normal spleen cells treated in identical manners.

(d) Spleen cells from mice with progressively growing P815 tumors (17-19 days after subcutaneous injection of $5X10^4$ P815 cells) were used as a source of suppressor cells.

TABLE XII. SUPPRESSIVE ACTIVITY IN LYMPHOID ORGANS FROM TUMOR BEARING MICE

Days after tumor injection[a]	Percent Suppression[b]		
	Spleen	Thymus	Lymph Node
4	7.8[NS][c]	18.7[NS]	7.0[NS]
8 8	-46.0[d]	61.2	-48.6
12	-595.1	53.9	- 5.0[NS]
16	-389.8	4.3[NS]	40.2
19	17.2	38.4	36.0

(a) DBA/2 mice received $5X10^4$ P815 cells subcutaneously. Spleens, thymuses or lymph noeds from 3 mice in each group were pooled and tested.

(b) Per cent suppression was calculated from the per cent decrease in total lytic units in the test cultures as compared to the controls.

(c) Difference in cytotoxicity is not statistically significant by t-test.

(d) Negative numbers show the enhancement of the cytotoxicity by tumor bearing lymphoid cells.

tumor cell injection, when the tumors were very small and spleen cells and lymph node cells showed some cytotoxic responses. The suppressive activity in thymus persisted in the stage of slow tumor growth (day 12) while spleen cells showed a strong immune response. Lymph node cells became suppressive (day 16) before the appearance of suppressor cells in spleens. When tumors grew progressively (day 19), suppressor cells were detected in spleens as well as in thymuses and lymph nodes.

Suppression by thymocyte extracts

Cell free extracts, prepared by freeze-thawing single cell suspension of normal or P815 tumor bearing thymocytes in PBS, were tested for suppressive activity. The thymocyte extracts were added to the *in vitro* cultures to generate specific cytotoxicity against P815 or L1210 cells. The extract prepared from thymocytes from P815 tumor bearing mice inhibited the generation of anti-P815 cytotoxicity as compared to the normal thymocyte extract (Table XIII). The same extract did not affect the generation of anti-L1210 cytotoxicity. Therefore the suppression of anti-P815 cytotoxicity by the thymocyte extract is specific and is not due to the presence of non-specifically toxic substances in the extract.

TABLE XIII. SUPPRESSION BY THYMOCYTE EXTRACTS

Cell Extracts[a]	Cytotoxicity[b] (%) ± SEM	
	anti P815[c]	anti L1210[d]
-	60.5 ± 2.6	38.2 ± 2.5
normal thymus	54.8 ± 2.7	47.1 ± 1.8
P815 thymus	43.6 ± 3.3	49.4 ± 1.3

(a) Thymocyte suspension in PBS ($5X10^7$/ml) was freeze-thawed three times aand centrifuged 20 min at 400 x g. Supernatant was filtrated through millipore membrane (pore size 0.2 μ).

(b) ^{51}Cr. release assay: Effector to target ratio was 40:1, incubation period was 18 hours. The figures are the means of triplicate tests.

(c) Spleen cells from DBA/2 mice bearing small P815 tumors (14 days after subcutaneous injection of 2 X 10^3 P815 cells) were used as P815 tumor immune cells to generate anti-P815 cytotoxicity.

(d) Spleen cells from DBA/2 mice bearing small L1210 tumors (10 days after subcutaneous injection of L1210 cells) were used as L1210 tumor immune cells to generate anti-L1210 cytotoxicity.

DISCUSSION

In preliminary studies, it was observed that spleen cells of DBA/2 mice, injected subcutaneously with a syngeneic mastocytoma cell line, developed specific cytotoxicity to their tumors during the first two weeks of tumor growth. The immunity could be measured by a direct assay. Cytotoxic cells were identified as T lymphocytes. Maximum cytotoxic activity coincided, in tumor bearing animals, with a period of slowed tumor growth, as assessed by direct measurement of the tumor size. Following this period, tumor growth resumed at an accelerated rate, and this occurrence coincided with a loss of specific cytotoxicity. The spleen cells of tumor bearing animals, at the time of loss of specific cytotoxicity, appeared to be normal in other functions such as their *in vitro* responses to mitogens. This observation was unlike previous observations made in this laboratory that the lymphocytes of DBA/2 mice with progressively growing transplantable methylcholanthrene induced sarcomas lost both tumor specific immunity and other immunologic functions such as mitogen responsiveness and the ability to respond *in vitro* to unrelated antigens (Whitney *et al.*, 1974).

In order to investigate this apparent suppression of specific cytotoxicity we established an *in vitro* method to induce cytotoxicity against the tumor. Unlike the methods reported by Wagner and Rollinghoff (1973) in which normal spleen cells were sensitized against syngeneic tumors, we used spleen cells from mice bearing small tumors which already demonstrated some cytotoxic activity against the tumor cells. Therefore, the response in these cultures was probably a secondary response, which facilitated detection, obviating the need for a complicated culture method. Similar methods to generate cytotoxicity against syngeneic tumors *in vitro* have been reported recently in other tumor systems including a mammary adenocarcinoma in rats (Kuperman *et al.*, 1975a),

a lymphoma (Glaser et al., 1976) and a methylcholanthrene induced sarcoma (Kall and Hellstrom, 1975) in mice.

The cytotoxicity induced in the present studies was specific to the tumor and mediated by T lymphocytes. Using this method, cells capable of inhibiting the in vitro generation of cytotoxicity were found in the spleen and thymus of mice at a later stage of the progressive growth of their tumors. Serum from mice with progressively growing P815 tumors inhibited neither the activity of cytotoxic cells nor the generation of them in vitro. It appears that the decline of cytotoxicity observed as the tumors progress can be attributed to the presence of suppressor cells which inhibit the generation of cytotoxic cells.

Characterization of these suppressor cells in P815 tumor bearing DBA/2 mice indicates that:

(1). Suppressor cells were sensitive to anti θ serum and complement treatment, but insensitive to anti mouse Ig serum and complement treatment or carbonyl iron and magnet treatment. Therefore, they seem to be T lymphocytes.

(2). Suppressor cells were specific to the tumor, i.e., suppressor cells from P815 tumor bearing mice did not inhibit the in vitro generation of cytotoxicity against another tumor line (L1210 leukemia in DBA/2 mice), nor did they inhibit the proliferative response of T lymphocytes to mitogens.

(3). Suppressor cells and killer cells migrate in separate fractions in ficoll-hypaque density cell separation. Therefore, suppressor T cells and killer T cells in this system appear to be distinct populations.

Suppressor cells that inhibit specific anti-tumor immunerresponses have been reported in other tumor systems. Suppressor cells from Japanese quails bearing Rous virus induced sarcoma (Hayami et al., 1972) and rats

bearing a mammary adenocarcinoma (Kuperman et al., 1975b) inhibited the generation of specific cytotoxicity against their respective tumors. The specificity and the nature of the cells involved was not tested in these studies. Suppressive activity of peritoneal cells from mice bearing sarcomas induced either by Moloney sarcoma virus or methylcholanthrene were reported by Halliday (1972). Using the macrophage migration inhibition assay, he found that peritoneal cells from mice with progressively growing sarcomas did not show an anti-tumor response. When these progressor cells were mixed with the cells from mice whose tumors had regressed spontaneously or had been surgically removed, they inhibited the anti-tumor response normally observed with the regressor cells. This effect was specific for the given tumor studied. A similar phenomenon has been reported by Fujimoto et al. (1976 a, b) in mice bearing methylcholanthrene induced sarcomas. Suppressor cells from these animals or their extracts, when injected into immune mice, decreased specific anti-tumor activity as determined by direct measurement of the tumor size in vivo. The suppressor cells were found in the thymus, spleen, lymph nodes and bone marrow of tumor bearing mice and were thought to be T lymphocytes because of their sensitivity to treatment with anti θ serum and complement.

Suppressor cells which inhibit general immune responses in a nonspecific way have been also reported in mice with sarcomas induced by murine sarcoma virus (Kirchner et al., 1974) or methylcholanthrene (Eggers and Wunderlich, 1975; Pope et al., 1976). Gorczynski et al. (1975) proposed that the suppressor cells in the former tumor system were B lymphcoytes which inhibited the generation of cytotoxici effector cells against tumor as well as the PHA response of T lymphocytes by means of antigen-antibody complexes. In contrast, Kirchner et al. (1975) suggested that the suppressor cell in the same tumor system were cells of the monocyte/macrophage series and that they inhibited

general immunity by inhibiting DNA synthesis in T lymphocytes. The nonspecific suppressor cells in a methylcholanthrene induced sarcoma system have also been identified as adherent cells of the macrophage/monocyte series (Pope et al., 1976) and inhibit general immune responses of normal lymphocytes such as the proliferative response to mitogens (Pope et al., 1976) or the response to allo-antigens in mixed leukocyte cultures (Eggers and Wunderlich, 1975). Although the relationship between nonspecific and specific suppressor cells in tumor bearing animals is not yet clear, it is thought that both of them can inhibit specific anti tumor immunity which may result in progressive tumor growth.

In the present study specific suppressor cells were detected in mice during the early stages of tumor growth when tumors were very small and some cytotoxic reaction was detected. Similar observations in a methylcholanthrene induced sarcoma were reported by Fujimoto et al. (1976 a, b) who detected specific suppressor cells in animals within 24 hours after tumor cell injection. On the other hand nonspecific suppressor cells in sarcoma bearing mice were normally detected in the later stages of tumor growth when the tumors grew progressively (Pope et al., 1976; Eggers and Wunderlich, 1975). Moreover, the cells involved in specific and nonspecific suppression seem to be different. Specific suppressor cells are thought to be T lymphocytes while nonspecific suppressor cells are not T lymphocytes (Kirchner et al., 1974; Pope et al., 1976). Therefore, these two types of suppression in tumor bearing animals may involve distinct mechanisms. It is possible that both types of suppression occur in many tumor systems, presumably in different stages of tumor growth. In fact, both types of suppressor cells have been detected in very similar tumor systems such as sarcomas induced by methylcholanthrene (Eggers and

Wunderlich, 1975; Fujimoto et al., 1976) or murine sarcoma virus (Kirchner et al., 1974; Halliday, 1972). The failure to detect non-specific suppression in P815 mastocytoma tumor bearing mice may be due to the fast growth and metastasis ofthe tumors.

Antigen specific suppressor T cells have been widely studied in various immunological systems (Gershon, 1975). It is generally assumed that these suppressor cells are directly involved in the regulation of immune responses. However, it is not known how suppressor activity is induced and in what way they inhibit immune responses. One of the approaches to these questions is to study relationships between various functions of T lymphocytes, i.e. helper, killer and suppressor. Recent studies on Ly antigens on subpopulations of T lymphocytes suggested that helper cells and killer cells are distinct populations, but killer cells and suppressor cells share the same Ly antigens (Medawar and Simpson, 1975). However, the present study suggests that suppressor cells and killer cells in P815 tumor bearing mice are not physiologically identical. When P815 tumor bearing spleen cells were fractionated by ficoll-hypaque density cell separation, killer cells were enriched in the fraction of heavier density while suppressor cells were found mainly in the lighter fraction. It is possible that they are in the same subpopulation of T lymphocytes but are in a different stage of cell differentiation.

The present studies on the development of suppressor cells at various stages of tumor growth indicate that suppressor cells develop in the thymuses of P815 tumor bearing animals at an early stage in tumor growth, while spleen cells are still demonstrating significant levels of cytotoxicity toward P815 cells. Since cytotoxic cells are never apparent in the thymus at any time during tumor growth, it seems unlikely that suppressor cells result from a further differentiation step of the specific killer cells.

These results also suggest a possible role of the thymus in immune responses in adult life. Although it has been well known that the thymus plays an essential role in T lymphocyte differentiation in neonatal life, the role of thymus in adult life has been unknown. Recent studies on the subclasses of T lymphocytes have shown that a population of T lymphocytes in the peripheral lymphoid system which seem to have a short life span are partially diminished by adult thymectomy. (Cantor et al., 1975). It has also been shown that adult thymectomy enhances various immune responses such as antibody formation against T lymphocyte independent antigens (Kerbel and Eidinger, 1972), primary cytotoxic responses to alloantigens (Simpson and Cantor, 1975) and the sensitization against syngeneic fibroblasts (Carnaud et al., 1975), suggesting that the short lived T lymphocytes that are sensitive to adult thymectomy are involved in the regulation of these immune reactions. Although the relationship between suppressor cells in thymuses, spleens and lymph nodes in P815 tumor bearing mice is not clear at this point, the present studies suggest that the thymus plays an important role in the suppression of anti-tumor immunity.

Although antigen specific suppressor cells have been studied in various immunological systems at the cellular level, their mechanisms are still largely unknown. Since specific suppressor cells seem to recognize antigens in a specific way, it is generally assumed that they also carry surface receptors for antigens and that the receptors are in some way involved in suppression. In the present study cell free extracts prepared from suppressive thymocytes had the same suppressive activity as the intact cells. Similar results have been reported by Fujimoto et al. (1976) using thymocytes from mice bearing sarcomas. Tada et al. (1975) also detected antigen specific suppressive factors in cell free extracts prepared from lymphoid cells which suppressed antibody formation both in vivo and in vitro. By using specific

antiserum against histocompatibility antigens, they demonstrated that the suppressive factors were associated with some histocompatibility antigens, most likely the products of I region genes.

Further studies using cell free extracts prepared from suppressive thymocytes in the P815 tumor system will enable us to study not only the tumor-host relationship in tumor bearing hosts but the regulation of the cellular immune response at the molecular level.

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