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Immunosuppressive Lymphocyte Factors: Characterization of the IDS-Producing Cell in the Experimental Model of Antigenic Competition

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Inhibitor of DNA synthesis, is a soluble, protein lymphocyte factor which nonspecifically suppresses *in vitro* lymphocyte responses to antigens or mitogens. It is secreted in large amounts *in vivo* in some experimentally induced immunological paralysis. Here, we have defined the cell secreting IDS in one experimental model of nonspecific immune-suppression, ie., that of antigenic competition.

Lymphocytes of rats injected with a large dose of ovalbumin intravenously, show no immunologic response to the same or other antigens or mitogens 24 hr later. At this time, spleen cells of these rats secrete large amounts of the inhibitor into culture supernatants. However spleen cell supernates of T-depleted rats do not contain the activity. Further, maximal inhibitor concentrations are obtained in the first 2 days of culture when more than 65% of cultured cells are large blasts actively synthesizing protein. As the number of actively metabolizing blast cells decrease in subsequent days of culture inhibitor concentration falls. Finally thymocytes of rats pretreated with hydrocortisone acetate, to deplete thymus cortex cells are unable to secrete inhibitor in culture.

These findings reveal that the cells producing inhibitory DNA synthesis in an animal made tolerant with a supra-optimal dose of antigen is an active blast transformed T cell (present in the spleen and thymus). In the thymus the cell making inhibitor appears to reside in the thymus cortex. Previous experiments have confirmed that an identical cell causes nonspecific immune-suppression *in vitro*. We suggest that this cell produces *in vivo* tolerance in antigenic competition through the release of inhibitor to DNA synthesis.

Inhibitor of DNA synthesis (IDS) is a soluble, protein factor secreted, *in vivo* and *in vitro*, by appropriately stimulated lymphocytes [1]. IDS suppresses the immunologic responses of antigen or mitogen-stimulated T and B lymphocytes *in vitro* [2,3]. The immunosuppressive effect of IDS is nonspecific in that it can inhibit the response of lymphocytes to antigens and mitogens which are different from the antigen that evoked its production [1]. Its inhibitory action is mediated through the elevation of cAMP in target cells [4,5]. IDS has recently been purified to homogeneity [6–8].

IDS is released in increased quantities by lymphocytes of rats injected intravenously with a large tolerogenic dose of a soluble protein antigen eg. ovalbumin (OA) [9]. These animals when tested later show a lack of immunologic response not only to the tolerizing antigen but also to other unrelated antigens, ie., they manifest nonspecific immune-suppression. This phenomenon where tolerization to one antigen results in total immune paralysis for a short period of time is called antigenic competition [10]. Since IDS is produced in increased amounts in such animals, its nonspecific immunosuppressive activity might be responsible for the immunologic unresponsiveness seen in these animals.

In this study we have defined the lymphocyte subpopulation which (when appropriately stimulated) secretes IDS in the experimental animal model of antigenic competition.

MATERIALS AND METHODS

Animals

Inbred male Lewis rats, 8–10 weeks old, purchased from Microbiological Associates, Bethesda, Maryland, were used throughout.

Reagents

Ovalbumin (OA) and phytohemagglutinin-P (PHA-P) were purchased from Difco, Detroit, MI, RPMI 1640 with penicillin and streptomycin from Associated Biomedics Systems, Buffalo, New York, NY Heat inactivated fetal calf serum (FCS) from GIBCO, Grand Island, New York [^{3}H]-thymidine, [^{14}C]-Amino Acid mix and Aquasol II from New England Nuclear, Boston, MA, and trypan blue from Bio Rad, Richmond, CA. Tissue culture flasks used were from Falcon plastics, Oxnard, CA, and the semi-automatic multiple-well cell harvester was purchased from Otto Hiller Company, Madison, WI.

Assay for IDS Activity

IDS activity was measured in each case, in crude supernatants and in supernatants purified to homogeneity as described previously [8]. 5×10^5 rat lymph node cells (LNC) are cultured in each well of microwell culture plates containing a total volume of 0.2 ml/well consisting of 0.1 ml of supernatants to be tested for IDS activity (see under preparation of supernatants), and 0.1 ml of RPMI 1640 with 2 μ 1/ml PHA and 10% FCS (final concentration). After incubation for 44 hr at 37°C and 5% CO_2, 1 μ Ci of 3H-thymidine (6.7 Ci/mM) is added to each well. After a further 4 hr, cells from each well are harvested onto discs of filter paper using the semi-automatic multiple-well harvester and TCA precipitable ^{3}H counts dissolved in aquasol are determined in an Intertechnique Liquid Scintillation counter. All results are expressed as an average of triplicate values. Standard errors of the mean are calculated from these values. % Inhibition of DNa synthesis is calculated using background counts (no PHA) as 100% inhibition and PHA stimulated (no supernatants added) 0% inhibition.

Animal Model for Obtaining IDS Containing Supernatants

The model used in our experiments is that of antigenic competition where nonspecific immune suppression by spleen cells has been previously demonstrated [11]. We have already shown the production of IDS by spleen and thymus cells in this model [9]. Briefly, normal male Lewis rats were injected intravenously with a large dose of ovalbumen (100 mgs in 1 ml of phosphate buffered saline). Control animals received normal saline. 24 hr later their spleens are harvested, minced and filtered to remove extraneous tissue. The spleen cell suspension obtained is pelleted and washed 3 times with RPMI medium. 5×10^6 viable nucleated spleen cells/ml were cultured in a total volume of 50 ml of plain RPMI 1640 (no added stimulant) at 37°C in an atmosphere

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Abbreviations:
FCS: fetal calf serum
IDS: inhibitor of DNA synthesis
LNC: lymph node cells
OA: ovalbumin
PHA-P: phytohemagglutinin

of 5% CO_2 in Falcon tissue culture flasks. Supernatants were harvested at 24 hr, concentrated 20 times and assayed for IDS activity.

Identification of Cell Secreting IDS in Above Model

Preparation of T-depleted rats: 6-week-old male Lewis rats were surgically thymectomized. One week later they were given 900 R total body X-radiation from a Siemans 10 kv X-ray machine and immediately reconstituted intravenously with 1×10^8 bone marrow cells (in 2 ml of normal saline) from normal syngeneic Lewis rats. When these rats were 7 weeks old they were sacrificed and their bone marrow cells used to reconstitute a newly thymectomized X-irradiated batch of syngeneic rats. Seven weeks later these rats were used as a source of B cells. Such rats have been previously demonstrated to be completely lacking in T cells by the criteria of absent *in vivo* cell mediated immunologic responses and impaired response *in vitro* to T cell mitogens [12].

Preparation of lymphoblast cultures: Spleen cells suspensions obtained from the antigen injected animals were adjusted to a concentration of 1×10^7 nucleated cells/ml in a total volume of 15 ml and layered on 10 ml of 9% Ficoll:39.9% Hypaque, 24:10 v/v_1 in a sterile polypropylene centrifuge tube. After centrifugation at 400 g for 20 min at 20°C in an RC 5 Sorvall centrifuge, the interface cells were collected and washed 3 times. These interface cells consist > 65% large blast cells as judged by the criteria of size and May-Grünwald-Giemsa staining characteristics [9]. The pellet of cells at the bottom of the gradient were also harvested washed well and the red cells lysed by standard hypertonic treatment. These cells consist mainly of medium size and small lymphocytes.

The 2 above cell types were cultured separately as before for production of supernatant. Supernatants were harvested at 24 hr intervals for 5 days and the cells recultured each day in an identical manner. The percentage of blast cells (by size and stain) and the viability of cells was determined prior to each reculture. Each supernatant was individually purified and assayed for activity.

Determination of protein synthesis by IDS producing cells: Small aliquots of the blast cells were obtained prior to each day of culture for these studies.

5×10^5 blast cells were cultured in microwell culture plates in a total volume of 0.1 ml RPMI 1640 with 10% FCS, at 37°C and 5% CO_2. After 12 hr 2.5 μCi/well of [^{14}C]Amino acid mix was added for a further 12 hr. The cells were then harvested using the multiple well cell harvester and [^{14}C]-incorporation into TCA precipitable protein determined in a liquid scintillation counter.

Determination of hydrocortisone sensitivity of IDS producing cell: Rats were pretreated with hydrocortisone acetate (150 mgs/kg body wt) intraperitoneally 48 hr prior to the intravenous injection of the large dose of antigen. 24 hr later the animals are killed, thymuses are harvested, teased and single cell suspensions of thymocytes are made. These are then cultured for production of supernatant as before. Thymuses of hydrocortisone treated rats yielded only about 21% of total cells obtained from saline injected control rats.

RESULTS

Table I depicts the inhibitory activity in purified supernates of spleen cells placed in culture 24 hr after injection of the large dose of antigen. Supernates of animals injected with ovalbumin produced almost complete inhibition of the DNA synthetic response of normal lymphocytes stimulated with PHA (83.6%) while spleen cells supernates of saline injected animals caused no inhibition (4.8%).

Rats, depleted of T cells by double thymectomy, X-irradiation and bone marrow reconstitution prior to injection of antigen, and injected with ovalbumin, behave like normal animals injected with saline (Fig 1). Their spleen cell supernates do not contain inhibitory activity demonstrating the necessity of T cells for production of factor.

Fig 2 shows the relationship of blast cells in culture (expressed as a percentage of total cells) to the production of inhibitory activity, on successive days of culture. In the first 2 days of culture the presence of 65–70% blasts in culture is associated with the secretion of maximal concentrations of IDS. By the third day of reculture a decrease in IDS production is seen with a reduction of blasts in culture. On the 4th and 5th days, cultures contain very few blasts and no IDS activity is seen. Viability of cells was 95% on the 1st two days, > 75% on the 3rd and 4th day and about 45% on the 5th day.

Animals injected with hydrocortisone acetate 48 hr prior to the injection of ovalbumin show a major loss of the ability of their thymus cells to produce IDS (Table II). Here supernatants from animals pretreated with saline cause 81.2% inhibition while hydrocortisone acetate treated animals have only 27.7% inhibitory activity. Since hydrocortisone depletes cortical thymocytes, cortex cells appear to be responsible for IDS production in the thymus.

TABLE I. *IDS activity in purified spleen cell supernatants*[a]

Supernatant source	DNA synthetic response of PHA stimulated normal LNC[b]	
	^{3}H-Tdr incorporation (CPM × 10^{-3})	Inhibition (%)
None	22,144 ± 918[c]	0
Saline injected donor	21,086 ± 1068[c]	4.8
OA injected donor (100 mgs/ml)	5504 ± 176[c]	83.6

[a] Spleen cells harvested 24 hr after donor treatment and cultured without added stimulant for 24 hr. Supernatants harvested and purified prior to assay.

[b] Standard IDS assay system.

[c] – Standard error [Background 2239 ± 96].

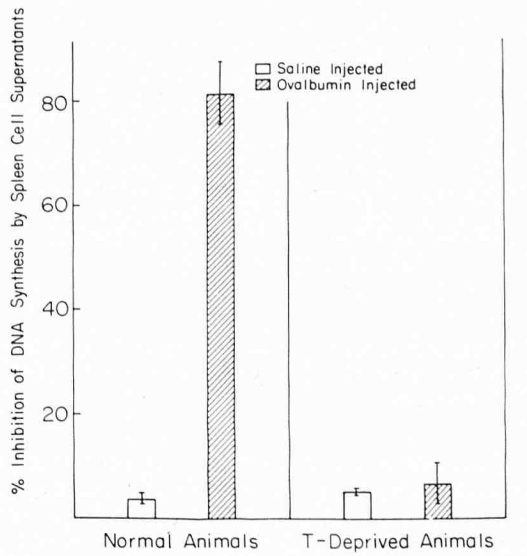

FIG 1. IDS activity in purified spleen cell supernatants. Spleen cells cultured 24 hr after intravenous injection of ovalbumin or saline. Supernatants harvested 24 hr after initial culture. IDS activity expressed as % inhibition of DNA synthesis (see Materials and Methods).

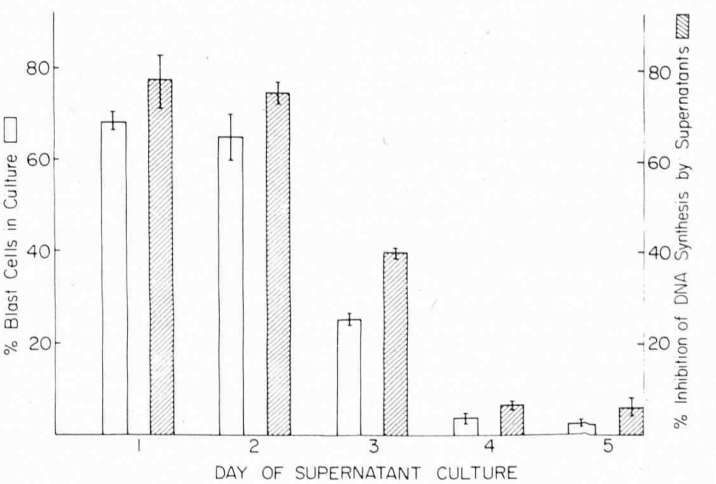

FIG 2. Relationship of blast cells in culture to production of IDS. Blast cells expressed as a % of total cells in culture.

TABLE II. *IDS activity in purified thymus cell supernatants of ovalbumin injected rats*

Pretreatment of animals	DNA synthetic response of PHA stimulated normal LNC[a]	
	^{3}H-Tdr incorporation (CPM × 10^{-3})	Inhibition (%)
Hydrocortisone acetate[b]	18,436	27.7
Saline[c]	4,794	81.2

[a] Standard IDS assay system.
[b] Hydrocortisone acetate (150 mg/kg) i.p., 48 hr prior to OA injection.
[c] Saline injected—same manner as *b*.

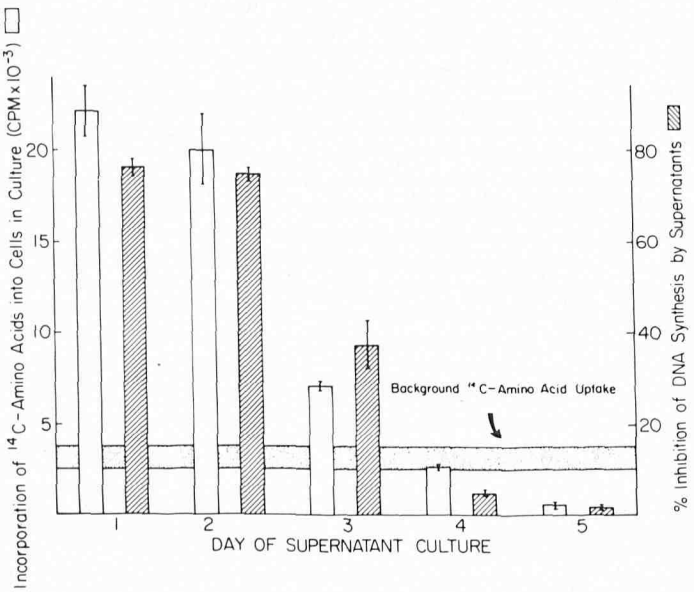

FIG 3. Relationship of IDS production to protein synthetic activity of cells in culture. Protein synthesis expressed at [^{14}C]Amino Acid incorporation into cells.

Finally Fig 3 demonstrates that IDS production is maximal when the cells in culture are actively synthesizing protein. Protein synthesis is measured as [^{14}C]Amino acid incorporation into cells. High inhibitory activity is seen on the first two days of culture when the cells are metabolically very active. As activity falls off from the third day onwards production of IDS decreases.

DISCUSSION

We have previously shown that IDS secretion by spleen cells of rats injected with a tolerizing dose of antigen occurs 24 hr after injection of antigens [9]. At this time the animal shows evidence of total immunologic unresponsiveness, thereby exhibiting the phenomenon of antigenic competition. We have also demonstrated that the subpopulation of cells releasing IDS in these immunologically paralyzed animals are adherent to glass [9]. Experiments by Bash and Waksman revealed that the cells causing this immune suppression were not macrophages [11].

Circumstantial evidence from earlier work had suggested that the cells making IDS were thymus-derived, ie., T cells. This premise was based on the fact that IDS was made by lymphocytes stimulated with a T cell mitogen, eg., Con A and that thymus cells, with appropriate stimulation, could secrete IDS [1,9]. In our present studies in the model of antigenic competition we have shown that spleen cells of animals depleted of only T cells, by experimental manipulation, are unable to make IDS. This finding adds confirmatory direct evidence to the previous implication that IDS is produced by T cells. A further finding was that maximal concentration of IDS was secreted 24 to 48 hr after *in vivo* stimulation, when the major proportion of ficoll-hypaque separated spleen cells in culture were large proliferating blasts. IDS production dropped off with a decrease in the number of blast cells in culture. We also found that thymocytes of these animals which normally secreted IDS 24 hr after the antigenic stimulus (like spleen cells) were unable to produce IDS if the animals were pretreated with hydrocortisone acetate which depletes cortical thymocytes. The above findings suggest the conclusion that a T cell subpopulation present in the spleen and thymus is capable of secreting IDS following an appropriate antigenic stimulus. In the thymus this IDS producing cell resides in the cortex of the gland. Morphologically the IDS secreting cell is a large blast.

Previous *in vitro* lymphocyte studies by Raff and Cantor [13] in mice demonstrated that nonspecific immune suppression was produced by T cells present in the thymus and spleen. Stutman [14] showed that the splenic cells causing this suppression were large blastoid T_1 cells, ie., T cells which had not acquired the θ (theta) surface marker. T_1 cells are normally not found in the peripheral circulation since T cells prior to entering the periphery migrate from the thymus cortex through the thymus medulla, where they acquire the θ (theta) antigen. Goldschneider [15] working with rats has shown that there is an alternate pathway of migration for T cells, directly from the thymus cortex to the spleen without passing through the medulla. Nonspecific suppressor T cells may migrate via this route. Our *in vivo* experiments in an animal model with nonspecific immune suppression show that the cell secreting IDS appears to be identical to the cell that has previously been shown to cause nonspecific suppression in vitro. Since IDS causes nonspecific inhibition of lymphocyte function it is possible that in these animals the immunologic tolerance is caused by IDS secreted by these suppressor cells.

A final finding was that IDS release into supernates occurred while the cells in culture were actively synthesizing protein. As protein synthesis decreased in subsequent days of culture IDS production diminished. However IDS was not produced in supernatants collected earlier than 20 hr of initial culture although active protein synthesis occurred and continued as early as 4–6 hr (data not shown). These findings suggest that IDS production requires live actively functioning cells. IDS therefore is not a product released passively by a dead or dying cell. Further, IDS release occurs later in the cell cycle, ie., in the stage of protein synthesis occurring just prior to or closely associated with DNA synthesis. This delayed release of IDS is very typical for the time sequence when the "immunoregulatory" lymphokines are usually produced in supernates.

In conclusion we have demonstrated that in animals with experimentally induced immune paralysis, IDS (an immunosuppressive lymphokine) is secreted by a unique subset of active T cells which are present in the thymus cortex and the spleen. These cells when appropriately stimulated secrete IDS which might be responsible for the immune suppression seen in these animals.

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DISCUSSION

GREEN: Can you find and isolate this factor (IDS) in human peripheral blood T cells?

JEGASOTHY: We can make it using Con A as a stimulant.

TURK: Have you looked at the effect of cyclophosphamide treatment on your system in rats?

JEGASOTHY: No we have not. You can sublethally irradiate these animals and still get the suppressive effect in these cells.

SCHILTZ: Will you comment on the tissue and species specificity of IDS?

JEGASOTHY: It works on lymph node cells. It also works on L cells which are transformed mouse fibroblasts.

SCHILTZ: You have tagged IDS as an inhibitor of DNA synthesis. Can you in fact differentiate whether the material inhibits DNA synthesis (perhaps directly) or whether it is an inhibitor of replication, for which DNA synthesis is merely a measured end point? Will IDS inhibit DNA synthesis *in vitro* with DNA template, triphosphates, enzymes, etc?

JEGASOTHY: We have measured the amount of DNA in the cells that are treated with IDS and the amount of DNA decreased. The amount of DNA is the same as in unstimulated lymphocytes.

BYSTRYN: Since your factor nonspecifically suppresses proliferation, do you think that conceptually it can be looked upon as a chalone of lymphoid tissue?

JEGASOTHY: You know we have talked about chalone being a suppressive factor. We have tried to find this factor from other cell types, stimulating other cell types, getting it from epidermal homogenates and we cannot find the same factor.

BRODY: Is this factor which you feel is responsible for antigenic competition only locally active or can it be active systemically? We found, in the 60's, that antigenic competition was an entirely localized phenomenon.

JEGASOTHY: I think that looking at serum factors is very difficult in the first place. I have done the same experiment you talked about and I cannot detect a circulating factor by the means we now have.

BERMAN: Was the anti-interferon antiserum you used anti-"immune" interferon or anti-"viral" interferon?

JEGASOTHY: We used both anti-immune interferon and antiviral interferon.

GIGLI: One wonders if this factor may bind to the lymphocyte membrane; therefore, it will not be found in the circulation.

JEGASOTHY: I think that is a great explanation and I think that is one of the reasons why many of the circulating factors cannot be actually found in serum.

CLAMAN: What is the relation of IDS to blastogenic factor? They are both made by activated T cells.

JEGASOTHY: We find that the IDS comes off at a different fraction than blastogenic or mitogenic factors do and we are able to separate out IDS pretty well.

Announcement

The XIth International Pigment Cell Conference will be held in Sendai, Japan, Oct. 14–17, 1980. The scientific sessions will be comprised of special lectures, symposia and workshops on pigment cell biology and human and animal diseases of pigment cells including malignant melanoma and leukoderma (vitiligo). Full details may be obtained by writing to Professor Makoto Seiji, Department of Dermatology, Tohoku University, Sendai, Japan. As in the previous 10 International Pigment Cell Conferences, there will be a multidisciplinary approach and participation will include biochemists, cell biologists, basic medical investigators and biophysicists.