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163

ANTIBODIES AGAINST HUMAN LYMPHOKINES: I. METHODS FOR INDUCTION OF ANTIBODIES CAPABLE OF NEUTRALIZING STABLE (α) AND UNSTABLE (β) LYMPHOTOXINS RELEASED IN VITRO BY ACTIVATED HUMAN LYMPHOCYTES

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Various methods were employed to induce antibodies in rabbits that were capable of neutralizing different families of lymphotoxins (LT). Both stable (α-LT) and unstable (β-LT) molecules, released by activated human lymphocytes in vitro, were neutralized. The different LT families were first separated into their respective groups by physical-chemical methods. Immunization with small quantities of antigen yielded a high percentage of responder animals. Techniques were developed for eliciting α-LT antibodies using as little as 2—3 ml of a cell-free supernatant. The situation was more difficult, however, when the unstable β-LT molecules were employed as antigens. We found that because of the low concentration and lability of β-LT in supernatants, the immunizing dose had to be: a) handled rapidly, b) larger than that used with the α-LT, and c) injected at closer intervals and over a longer immunization protocol. Physical-chemical studies supperted the concept that the LT neutralizing activity in the immune serum was immunoglobulin.

INTRODUCTION

Lymphoid cells from experimental animals and man when grown in vitro, can release a complex array of effector molecules termed lymphokines (LK). Lymphokines are synthesized and released when lymphocytes are specifically activated by: contact with antigens, mixed lymphocyte culture, or non-specifically by co-culture with mitogens (Dumonde et al., 1969; Granger, 1972). One group of LK, the lymphotoxins (LT), can cause either cytolysis or inhibition of the growth of cells in vitro (Ruddle and Waksman, 1967; Granger and Kolb, 1968; Jeffes and Granger, 1976; Namba and Waksman, 1976). Physical-chemical studies reveal that LT secreted by activated human

lymphocytes is composed of a complex group of materials divisible into at least three families. By differences in their molecular weight, stability, and kinetics of appearance in culture, these families have been designated the alpha, beta and gamma (Hiserodt et al., 1976; Walker et al., 1976). These substances are highly active and are present in very low concentrations in lymphocyte supernatants. The lymphotoxins have been proposed to be important effectors in cell and tissue destructive reactions induced by lymphoid cells. Their actual role in these reactions, however, is still uncertain.

A major objective in the study of these substances has been to develop antibodies which would specifically interact with LT molecules and thus provide a means to elucidate their role in various manifestations of cell-mediated immunity in vitro and in vivo. The present report describes methods for the induction of antibodies in rabbits that possess in vitro reactivity against stable (alpha) and unstable (beta) human LT. New immunization approaches requiring small amounts of immunogen, result in a high percentage of responding animals and can result in reactive antisera, in certain cases, within 6 weeks after immunization.

MATERIALS AND METHODS

Lymphocyte culture and supernatant collection

Lymphocyte suspensions were obtained from human tonsils and adenoids, and established in culture as previously described (Lewis et al., 1976). Cells were maintained at a density of 5×10^6 cells/ml in Hanks minimal essential media (MEM) containing 20 μg/ml phytohemagglutinin-P (PHA Difco, Detroit, Michigan), 10 to 20 μg/ml of a heat-stable boiled serum fraction obtained from newborn calf serum (BS), antibiotics and glutamine. Supernatants containing LK were collected from these cultures 3 and 5 days later. To obtain totally protein-free supernatants, we employed a modification of the method reported by Boulos et al. (1974). Briefly, lymphocytes were cultured in the same medium as described above with 5% fetal calf serum (Grand Island Biological Laboratories, Grand Island, New York). After 48 h, the medium was discarded, the cells washed twice in MEM and established in MEM with all the above additives except there was no BS added and only 10 μg/ml PHA-P was used. Supernatants were collected from these cultures 24 h later, because lymphoid cell viability rapidly declined after this interval. All supernatants from activated lymphocytes (SAL) were cleared of cells by either centrifugation at 300 g for 10 min, or passage through one layer of glass fiber filter paper (Gelman, Ann Arbor, Michigan), in a Buchler suction funnel. To prevent LK adherence to this filter, it was pre-washed with 20 ml of a 0.05 M NaCl solution buffered with 1.5×10^{-3} M phosphate, pH 7.2 (PBS) containing 1% bovine serum albumin (BSA). Cell-free supernatants were pooled and either frozen or used immediately.

Immunogen preparation and physical-chemical methods

All supernatants were first concentrated by a factor of 30, 50, or 100 by passage through a BioRad hollow fiber device with a 30,000 MW exclusion size at 4°C. The concentrate was aliquoted and frozen at −70°C and/or subjected to a sequence of various physical-chemical separation procedures, most of which have been previously described (Kolb and Granger, 1968; Granger et al., 1973). Diethyl aminoethyl (DEAE) cellulose chromatography was performed on SAL at pH 8.0 in a 0.01 M Tris, 0.1 mM EDTA, 0.025 M NaCl, employing a linear eluting salt gradient of 0.025 to 0.035 M NaCl. Single step separation of IgG from NH_4SO_4 fractions of whole rabbit sera were performed in the same buffer in 0.025 M NaCl. Sephadex chromatography was conducted in PBS or 0.15 M NaCl, 0.01 Tris, pH 8.0 Polyacrylamide gel electrophoresis was by the method of Davis (1964), with a 1.0 cm stacking and a 7 cm separating gel at pH 9.6. Isoelectric focusing was conducted in an LKB 110 ml column, employing a 1% or 4% ampholyte solution with a pH gradient from 5—11 or 6—8 for 48—72 h. Small samples, usually 0.1 to 0.01 ml, of fractions collected from each of the above procedures were tested for LT activity by adding directly to 1.0 ml cultures or target L cells. Fractions containing the desired LT activities were pooled and concentrated, either by passage through Amicon filtration membrane of 10,000 or 30,000 MW pore size or by lyophilization. Protein concentrations were determined by the method of Lowry et al. (1951). Micro-Ouchterlony double-diffusion tests were performed on microscope slides coated with 3.0 ml of 1.5% agarose in PBS with 0.02% sodium azide. Immunoelectrophoresis was done on agarose-coated slides in barbital buffer, pH 8.6. Sepharose 4B was activated and proteins bound by the method of March et al. (1974). Proteins separated by affinity chromatography were eluted in 2 M NaI, pH 9.0 (Avrameas and Ternynck, 1967).

Lymphotoxin assay

The details of these techniques have been described previously (Spofford et al., 1974). Monolayers of indicator L cells were preestablished in 16 × 125 mm glass screw-capped culture tubes at a density of 10^5 cells/ml in MEM + 3% fetal calf serum containing 0.5 μg/ml of mitomycin C (Sigma Chemical Co., St. Louis, Missouri). Test and control samples were diluted by five-fold dilutions in MEM + 3% FCS and added to the cultures. After 24 h, viable cells were enumerated in a Coulter Counter. A unit of LT is defined as the quantity of LT which gives a 50% reduction of target cell numbers. The total LT units in a given sample is obtained by taking the reciprocal of the last dilution effecting a 50% reduction.

Immunization procedures

All experiments employed random-bred female New Zealand white rabbits (Valley Lab Supply Co., Mira Loma, California), 2.5 to 3.0 K in weight. Animals were maintained in standard vivarium conditions with food and water ad libidium. Samples for injection were emulsified in an equal volume of Freund's complete adjuvant (FCA). Acrylamide gel samples were first macerated by passage through a 3.0 ml syringe, then PBS was added, and the suspension repeatedly passaged through a 20 gauge needle. The mixture was then emulsified in an equal volume of FCA. Animals were immunized by three basic routes — subcutaneous (SC), intralymph node (IN), and intradermal (ID). The former were administered at the nuchal region and directly into the popliteal lymph nodes. The latter were administered by the method of Vaitukaitis et al. (1971). Briefly, hair was removed from each side of the back. Approximately 0.05 ml of emulsion was injected ID at 20 different sites over the entire back of the animal using a 1.0 ml tuberculin syringe and 23 gauge needle. In addition, 0.5 ml of *Bordetella pertussis* (E. Lilly Corp., Indianapolis, Indiana) was injected S.C. into two separate sites in the shoulder and a small area of the thigh.

Collection and handling of sera

Animals were bled by cardiac puncture, whole blood was transferred to 50 ml screw-capped centrifuge tubes and allowed to clot at $37°C$ for 2 h. The supernatant was carefully aspirated and cleared of RBC by centrifugation at 500 g for 15 min. Cell-free serum was heated at $56°C$ for 45 min, filter-sterilized and frozen at $-20°C$ in 7.0 ml aliquots. Pre-immune serum was collected from each rabbit before beginning an immunization protocol.

RESULTS

Assessment of the capacity of sera from normal and immunized rabbits to inhibit human LT induced L-cell cytolysis in vitro

Several methods of measuring sera for LT-inhibiting activity in vitro were tested. One technique was selected and employed in all three studies. It consisted of the following steps: 0 to 200 μl of test or control sera were incubated with 1 ml of the LT sample for 1—2 h at $37°C$. The sample was then serially diluted in MEM + 3% FCS, and 1 ml of each dilution tested on triplicate L-cell tube cultures for cytotoxicity. The effect of test and control sera on the lytic LD_{50} endpoint of the sample was determined and could be directly related to the number of units of LT neutralized. The data from an experiment shown in fig. 1, in which 100 μl of anti-alpha-LT was tested on a standard SAL containing α-LT illustrate this procedure. It can be seen that 100 μl of the test sera from rabbit A9 inactivated 95% or 762 units of α-LT
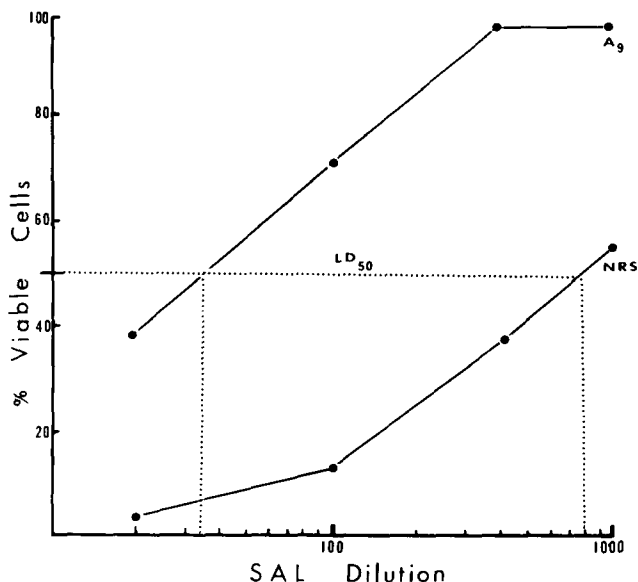
Fig. 1. The capacity of sera from normal and immunized rabbits to inhibit human alpha-LT induced L cell cytolysis in vitro. Test and control sera (100 μl) were added to a standard SAL with a high level (800) units of alpha-LT activity. After incubation for 1 h at 37°C, serial dilutions were tested for LT activity on target L cells, as described in Results. The LD_{50} endpoint of cytolysis was then determined, and the units of activity neutralized, calculated by:

$$\frac{\text{Titer of normal rabbit serum minus titer of immune serum}}{\text{Titer of normal rabbit serum}} \times 100 = \% \text{ activity neutralized.}$$

as compared to the normal rabbit serum (NRS) control. We found little or no difference between the effect of NRS and pre-immunization serum from the same animal. It was routinely observed that serum from certain unimmunized animals was able to cause a reduction of 3—15% of detecable LT activity. These sera had no detrimental effect on the target L cells.

Experiments were also conducted to determine the effectiveness of the different sera to neutralize various LT preparations. In these tests, the amount of LT was held constant, and increasing amounts of serum were added to 1 ml LT samples. After 1—2 h at 37°C, the samples were tested directly for LT activity. The results of several of these tests employing different antisera and crude and refined LT preparations are shown in fig. 2. All the test sera had inhibiting effects on in vitro lytic activity, which reached a maximum value at ~100 μl and then leveled off when tested against standard preparations containing 100 to 300 units of LT/ml. Three levels of each serum, 50, 100, 200 and 300 μl were compared for neutralizing activity against standard LT preparations containing between 100 to 300 units/ml of activity. Individual bleedings from all animals were stored and tested separately. Standard lots of supernatants were employed when testing antisera directed against stable α-LT activity; however, only fresh supernatants or
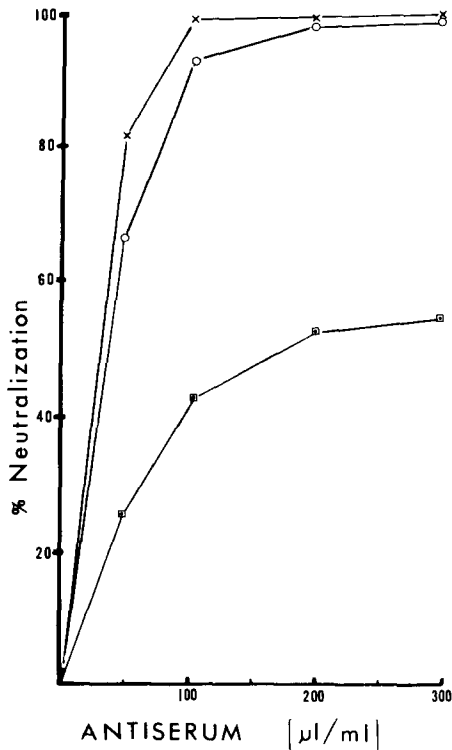
Fig. 2. The capacity of various amounts of test sera used to inactivate constant levels of LT activity on L cells in vitro. Increasing amounts of test and control sera were added to 1 ml samples of either whole SAL or sephadex fractions containing alpha-LT activity. They were incubated 1 h at 37°C and duplicate samples tested directly on L cells as described in Results. The degree of neutralization was determined in comparison with the effects of similar amounts of normal or preimmune serum controls as described in fig. 1: a) antisera from rabbit D4 immunized with whole SAL tested on fresh unfractionated SAL containing both α and β LT (X—X—X—X); b) antisera from rabbit A9 immunized with alpha-LT tested on sephadex fractions containing only alpha-LT activity (O—O—O—O); c) antisera from rabbit A1 immunized with alpha-LT tested on fresh unfractionated SAL containing both α and β LT (□—□—□—□).

column fractions were employed when testing antisera directed against the unstable β-LT activities.

Induction of antisera capable of neutralizing members of the α-LT family of human lymphotoxins released in vitro by PHA-activated human lymphocytes

Human α-LT are a family of stable molecules of approximately 80,000 MW. Fractions employed for injection of animals in this first series of studies were obtained from the last two stages in a separation protocol, which consisted of the following sequential steps: a) 5 day SAL concentrates were

passed through a DEAE column (2.4×30 cm), and the major peak of activity eluted off the column at the beginning of the gradient was collected, concentrated and applied to a Sephadex G-150 column (2.4×100 cm); b) the major peak of activity eluting in the 60,000 to 90,000 MW range was collected, this fraction pooled, concentrated and subjected to c) isoelectric focusing in a pH 5—11 gradient; d) the activity that focused at pH 7.0 to 7.4 was collected, concentrated and stored at $-70°$C. Individual animals were injected with samples containing an average of 3,000 units of activity from either the gel filtration or isoelectric focusing steps. Ten (2.5—3.0 K) female New Zealand white rabbits were immunized by several different routes and schedules. These rabbits were identified as the A series, 1—10. Four rabbits (A3, 4, 7 and 8) were immunized subcutaneously (SC) with six injections at two sites in the nuchal region and a small area of the thigh, every ten days. The animals were bled 10 days after the last injection, and boosted every 30 days and once every two weeks thereafter. In addition, two rabbits, A9 and 10, were immunized in the manner described above, using LT from a sephadex fraction that had been further purified by polyacrylamide gel electrophoresis (rf 3.0—3.5). Booster injections were given every 30 days using approximately 3,500 units of LT/animal, mixed as before, and injected in a similar manner. The remaining four rabbits (A1, 2, 5 and 6) were immunized by the ID-Vaitukaitis technique. They received SC booster injections once every 30 days, six weeks after the primary injection. Eight of the ten animals injected had responded by 20 weeks; these included all of the animals injected by the Vaitukaitis method and 2/3 of the animals injected subcutaneously. The animals which responded strongly became positive at 10 to 16 weeks, and the amount of inhibiting activity remained at high levels for the duration of the experiment (50—100 weeks). The results of testing the inhibiting activity in samples collected from these animals at either 20, 30 or 40 weeks, and tested against the standard α-LT containing SAL, are shown in table 1. The sera collected from rabbits A1, 4 and 9 were effective in inactivating α-LT activity. Additional testing revealed that 1 ml of antisera was capable of neutralizing up to 6,000 units of LT activity. It will be shown in later manuscripts that rabbits A1 and 9 exhibit no cross-reactivity with the unstable β-LTs, while serum collected from rabbits A4 and 5 show considerable cross-reactivity.

The above studies required large amounts of LT and a great deal of effort; therefore, experiments were designed in attempts to elicit inhibiting activity in rabbits employing much smaller amounts of immunogen. LT samples for injection in the next series of animals (B series) were prepared in the following manner: multiple samples of a standard SAL concentrate (30×) containing predominantly α-LT were subjected to polyacrylamide gel electrophoresis and immediately frozen at $-70°$C. One gel was cut into 1-mm slices, and each slide eluted overnight in 0.3 ml MEM, and 0.1 ml of the eluate tested directly for LT activity. Multiple peaks of activity were detected; however, the major peak of α-LT activity migrated with an R_f value of 0.3 to

TABLE 1

The capacity of sera from rabbits immunized with large doses of human α-LT to inhibit LT induced lysis of L cells in vitro *.

A series animals	Method of primary immuniza-tion **	Number of injections (total units of LT injected $\times 10^5$)	No. days after primary injec-tion	Amount of neutraliza-tion ***
1	ID-VK	8 (27.5)	271	+++
2	ID-VK	8 (27.5)	271	++
3	SC	12 (39.0)	271	—
4	SC	12 (39.0)	271	+++
5	ID-VK	7 (24.2)	271	+++
6	ID-VK	7 (24.2)	271	+++
7	SC	12 (39.0)	271	—
8	SC	11 (35.5)	232	+++
9	SC	9 (18.5)	143	+++
10	SC	7 (21.5)	143	+

* Female New Zealand white Rabbits were injected via different routes with fractions containing predominantly human α-LT. Sera was collected and tested for inhibiting activity on L cell cultures treated with a whole SAL which contained predominantly α-LT.
** ID-VK, intradermal-Vaitukaitus; SC, Subcutaneous.
*** Neutralization refers to inactivation of 200—300 units if LT activity by 100 μl of antisera calculated by:

$$\frac{\text{Units in normal rabbit serum minus units in immune serum}}{\text{Units in normal rabbit serum}} \times 100 = \% \text{ activity neutralized}$$

— = 0—15% neutralization; + = 15—40% neutralization; ++ = 40—80% neutralization; +++ = 80—100% neutralization.

0.35 with regard to the reference bromphenol blue marker dye. By direct testing, it was determined that this gel segment contained approximately 100 units of LT activity. The remaining frozen gels were sliced and the segments corresponding to R_f 0.3—0.35 were pooled, crushed into small fragments, and homogenized in FCA. The total sample obtained from 10 gels was injected into 5 rabbits by the ID-Vaitukaitis technique described in Methods. The animals were bled, and the sera tested against standard SAL 49 days after the primary injection. Each animal was then boosted with 3 injections over a period of 60 days, with 100 units of LT in FCA, subcutaneously in two granuloma sites on opposite sides each time. The LT employed for this booster was prepared in the following manner: The SAL concentrate was collected and passed sequentially through two Sephadex G-100 columns (4.5 × 100 cm and 2.4 × 100 cm) to remove all traces of β-LT. The fractions were collected, tested and mixed with FCA for injection in the manner described above. The animals were bled 5 days after the last injection and the serum tested for inhibiting activity. The results of these tests are shown in table 2. It is apparent that within six weeks after one immunization, one

TABLE 2

The capacity of sera from rabbits immunized with small doses of human α-LT to inactivate LT-induced cytolysis of L cells in vitro *.

B Series animals	LT inhibitory activity of serum collected	
	After single injection (42 days)	After secondary booster (112 days)
1	+++ **	+++
2	—	+++
3	—	+++
4	—	+++
5	—	+++

* Partially purified human alpha-LT was injected by the ID-VK technique into each animal as described in Methods. Sera from each animal were collected 6 weeks after a single injection and 10 days after the last of three secondary injections of 200 units administered SC 20 days apart.
** As in table 1.

of the five animals was responding. However, after the booster immunizations all animals were responding. At this time, each animal had received a total of only 800 units of α-LT.

Induction of antisera capable of inactiving short-lived LT molecules released in vitro by PHA-activated human lymphocytes

While the situation is complex, there are at least two families of human LT molecules present in a given supernatant from PHA-activated human lymphocytes, termed alpha and beta (Hiserodt et al., 1976; Walker et al., 1976). Alpha and beta lymphotoxin can be separated by sephadex chromatography on the basis of molucular weight differences, i.e. alpha 80,000, and beta approximately 50,000 daltons. The former are relatively stable while the latter generally have a very short half-life in serum-containing medium, even at 4°C. We have recently shown that the beta family consists of multiple molecules and have found methods to separate and partially stabilize them (Hiserodt et al., 1976; Hiserodt and Granger, 1976). Fractions containing all the β-LTs were prepared in the following manner: SAL concentrate (30×) was rapidly and sequentially chromatographed on two G-100 columns, the first 4.5 × 100 cm, the second, 2.4 × 100 cm. Fractions were tested and pooled to eliminate all traces of α-LT (Hiserodt and Granger, 1976). After testing, the fractions were immediately pooled, concentrated and emulsified with FCA, and 100 units of LT injected into four separate sites on 5 animals (C series), one on each flank and into each popliteal lymph node. Thereafter, animals were injected in an identical fashion, nine times over the next 23 weeks, with freshly isolated fractions. The animals were

bled for the first time, five days after the fourth injection. All sera were tested against both fresh 3-day SAL and sephadex fractions containing β-LT activity. The results of antisera tested on fresh 3-day SAL, containing 200 units of β-LT and sephadex refined β-LT fractions, are shown in table 3. After four injections, three of the five animals immunized responded, but only weakly.

Induction of polyspecific antisera directed against whole unfractionated serum-free SAL from PHA-stimulated human lymphocytes

Because the above methods did not result in induction of sera with high levels of neutralizing ability, we decided to try other techniques. In addition, the identification of short-lived effector molecules in these SAL suggested the possibility that there may be lymphokines so labile that they may never survive even simple fractionation procedures. We therefore decided to induce antisera that might possess inhibiting activity against all lymphotoxins present in whole SAL. Fractions for injection were handled as quickly as possible and prepared in the following manner: Human lymphoid cells were established for 48 to 72 h in MEM containing 5% fetal calf serum. They were then washed thoroughly and passed into RPMI 1640 medium with PHA for 24 h. At the end of 24 h, the media were collected, immediately concentrated 50X through a PM-10 membrane, emulsified in FCA, and the equivalent of 80 ml SAL was injected subcutaneously in each flank and directly into each popliteal lymph node for a total of eight injections, at 7-day intervals. These fractions contained from 200—300 units of each α and

TABLE 3

The capacity of sera from rabbits immunized with unstable human β-LT to inactivate whole SAL and β-LT induced lysis of L cells in vitro *.

C series animals	Method of primary immunization	Response against	
		Whole SAL	β-containing fractions
1	SC **	+ ***	+
2	SC	+	+
3	SC	—	—
4	ID-VK	+	+
5	SC	—	—

* Animals received injections of sephadex fractions containing approximately 100—200 units of human β-LT activity. Sera were collected after multiple injections and tested for inhibiting activity against whole fresh SAL and sephadex fractions containing only β-LT activity as described in fig. 1.
** As in table 1.
*** As in table 1.

TABLE 4

The capacity of sera from rabbits immunized with concentrated protein-free SAL from activated human lymphocytes to inactive LT activities in fresh SAL in vitro *.

D series animals	Response after 61 days **
1	+
2	+++
3	—
4	+++
5	—

* Animals were injected with freshly concentrated whole supernatants once a week for 8 weeks as described in Methods. Sera were collected 5 days after the last injection and tested on L cells treated with fresh SAL as described in fig. 1.
** As in table 1.

βLT. The animals were bled five days after the last injection and tested for activity against fresh whole supernatants and column fractions. The results of these studies are shown in table 4. The serum from two of the five animals in this series (D) was capable of completely neutralizing all α and β-LT activity.

TABLE 5

LT-inhibiting activity in sera of immunized animals identified as immunoglobulins. Sera from animals A1, 4 and 9, B2, C2 and C4 and D2 and D4 were subjected to physical-chemical and various immunologic tests to determine the nature of the inactivating material. The details of these tests are presented in Materials and Methods.

Treatment	Effect on neutralizing capacity of the preparation
1 33% NH$_4$SO$_4$	Total activity in ppt.
2 Absorption of whole sera (WS) or NH$_4$SO$_4$ ppt. with an excess of: (1) L-cells, (2) human lymphocytes, (3) PHA	No effect
3 Step DEAE chromatography of NH$_4$SO$_4$ ppt. (pH 8.0 — 0.025 M NaCl)	50—80% of activity isolated in IgG fraction
4 Immunoelectrophoresis of DEAE fractionated active component	Migrates as IgG uncontained with other serum proteins
5 Affinity chromatography of WS over goat anti-rabbit IgG bound to Seph. 4B Elution of column with 2.0 M NaI pH 9.0	Removal of 50—80% of activity Recovery of activity
6 Passage of SAL over Seph. 4B affinity column with rabbit anti alpha-LT IgG Bound Elution with 2.0 M NaI pH 9.0	Removal of 50—90% of lytic activity Recovery of 70—95% of bound activity

Physical-chemical and immunologic identification of the inactivating materials in sera collected from immunized animals, as associated with IgG

The sera from these animals has been subjected to a variety of physical and immunologic methods to identify the molecular characteristics of the inhibitory material. A summary of the tests is shown in table 5. The studies have been performed on the sera obtained from rabbits A1, 4 and 9, B2, C2 and 4. Physical studies indicate that the material in these sera is macromolecular, precipitates in 33% NH_4SO_4, and 50—70% separates on DEAE as immunoglobulin G. The DEAE fractions were subjected to analysis by immunoelectrophoresis and double diffusion. The material(s) in the DEAE fraction migrated as a single band when tested against goat anti-whole rabbit serum and anti-IgG. In addition, treating the sera with an immunoabsorbent prepared from Sepharose 4B-bound goat anti-rabbit IgG removed most of the neutralizing activity which could be eluted from the affinity column. Absorption of these antisera with either lymphocytes, L cells, or Sepharose 4B-bound PHA, and bovine serum did not diminish their neutralizing capacity. Finally, the nature of the LT inactivation itself is readily reversible by techniques which dissociate Ag-Ab reactions. These latter studies, with rabbit anti-human α LT IgG affinity columns, employing sera from rabbit A1 and 9, have been described elsewhere (Fair and Granger, 1976).

DISCUSSION

There is an obvious need for the development of dependable methods for the induction of antibodies against lymphokines to aid in their identification and separation, and to define their roles in immunologic reactions, both in vivo and in vitro. It has been difficult to study systematically individual lymphokines, because a whole supernatant medium possesses a spectrum of effector molecules with varying degrees of stability and longevity. A limited number of studies in other laboratories have resulted in reports on the production of antibodies against human lymphotoxin (Walker and Lucas, 1974; Jeffes and Granger, 1976), guinea pig MIF (Geczy et al., 1976), mitogenic factor (Gately et al., 1975), and whole unfractionated guinea pig SAL (Yoshida et al., 1974). These studies employed small numbers of animals without various immunization protocols. The previous reports dealing with antibodies against human LT involve long immunization procedures, only three animals, and high levels of lymphotoxin/injection, similar to the A series of animals reported in the present study. There is a distinct need for better defined methods which will require smaller amounts of immunogen and a larger number of responder animals.

The major goals of the present study were to develop efficient methods for the induction of antibodies against both stable and labile members of the human LT cytotoxin family. We employed both traditional techniques in the A series animals, which required considerable effort. These studies involved

the generation of 40 liters of SAL and all the associated concentration, fractionation, and separation processes.

Each animal received a total amount of from 18,500 to 40,000 units of LT activity. However, we found in the experiments with the B series animals that we could induce credible responses with less material. These animals were immunized with a total of 800 LT units and in one instance antibodies were induced with one injection of 200 units of LT activity. This is approximately 1—3 μg of LT protein, from 50 to 200 times less material than that employed for the A series animals. The routes and timing of immunization were critical in these studies, and the ID-VK method followed by subcutaneous booster injections appears to be the most reproducible and requires the least amount of material to elicit a response against the stable alpha-LT molecules. In addition, this method was successful employing both soluble and acrylamide gel fractions.

The induction of antibodies against the relatively short-lived members of the β-LT family is more complex. These fractions were injected subcutaneously or ID-VK, and generally resulted in a weak response, evidenced by the C series animals. However, it is clear that these materials are immunogenic, as shown by the marked response of the animals to injections of whole concentrated SAL in the D series. The differences between the C and the D series were as follows: 1) the immunogens were administered at more closely spaced intervals, once a week, 2) they were given in higher doses, and 3) they were handled rapidly after collection from the lymphocyte cultures. Thus, the critical feature appears to be repeated exposure and rapid handling to preserve maximum antigenicity of these particular lymphokines.

The inactivating materials in the sera from immunized animals have both the physical and immunological properties associated with immunoglobulins, as evidenced by: a) fractionation of whole serum by ammonium sulfate and ion exchange chromatography on DEAE, b) immunological identification of these substances as IgG proteins, and finally c) the inactivation process itself, which is completely reversible under the conditions dissociating antigen—antibody complexes. In particular, these latter studies discount the possibility that inactivation is due to an enzymatic degradation.

The substances associated with LT cytotoxicity in vitro consist of complex families of stable and unstable macromolecules. The alpha family contains at least three major subgroups (Lee and Lucas, 1976), and while not reported here, we have evidence indicating that these subgroups may be immunologically distinct from one another. The beta family has been fractionated into two submembers that are unstable. A third family, called the gamma family, has been identified, but at present little is known about the physical properties of these materials. The present studies were directed at inducing antibodies against whole families, rather than subfractionated members of individual families. These efforts resulted in the generation of large quantities of sera with activities which inhibit the various human lymphocyte lymphotoxins. The effects of the purified IgG fractions on different LT

molecules, lymphocytes, and direct cytotoxicity reactions, will be the topic of further publications.

ACKNOWLEDGEMENTS

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