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Biology, Biochemistry, Clinical Biochemistry
Volume 3

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Bern, May 31 – June 5, 1982

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Preface

At the end of the Fourth Lectin Meeting in Copenhagen in June 1981 we decided that future meetings should be organized at varying locations and on a collaborative basis. Therefore, from May 31 through June 5, 1982, the Fifth International Lectin Meeting 'INTERLEC 5' was held in Switzerland in cooperation of the Universities of Copenhagen and of Bern.

Over 100 participants attended the following sessions: Detection of Cancer Markers; Biological Function and Biological Effects of Lectins; and Lectins as Specific Tools. Eighty five communications (lectures and poster presentations) were given, the majority of which appears in this volume. Although the scope of topics in the present book is wide again - as it should be from a meeting covering the whole range of recent progress in the rapidly developing field of lectins - it is noteworthy that a great many contributions were centered on interactions of lectins with normal or malignant cells, opening new and exciting aspects for the application of these natural tools, e. g. in transplantation immunology and tumor diagnosis.

In addition to the scientific sessions, the Third Meeting of the International Working Party on Standardization of Lectins for Diagnosis was held, and a test program for con A from different sources was set up.

After four successive meetings in Copenhagen, it was indeed a challenge to organize 'INTERLEC 5' in Bern. It was for the large number and the scientific quality of the presentations, as well as for the high spirits that prevailed throughout the entire meeting that this event was particularly rewarding and unforgettable for the organizers. We wish to express our sincere appreciation to all participants of 'INTERLEC 5' and to thank Dr. B.Nordbring-Hertz, Lund, for her helpful assistance in planning the meeting.

'INTERLEC 5' was generously supported by the following institutions and companies (in alphabetical order): The Authorities of the State and of the City of Bern; Behringwerke AG, Marburg, Lahn; Boehringer Mannheim GmbH, Hamburg; Hoechst-Pharma AG, Zurich; Medac GmbH, Hamburg; Miles Italiana S.p.A., Cavenago Brianza, Milano; Riact, Copenhagen. Their help has been greatly appreciated.

'INTERLEC 5' will be held in 1984, and we look forward to meet you again at our next lectin meeting.

Copenhagen and Bern, January, 1983

T.C.Bøg-Hansen and G.A.Spengler

Obituary

During preparation of this volume, we recieved notice that one of the devoted participants of the INTERLEC meetings had unexpectedly deceased. Prof.Dr.med. Gunther Hermann, Docteur de l'Université de Paris, mention Sciences, Chief of the Immunological Division at the Surgical University Hospital in Cologne, was born September 20, 1924, in Stuttgart and died August 20, 1982, in Avignon.

We mourn for Gunther Hermann and we shall always remember him as a distinguished scientist and a devoted friend of the lectinologic community.

Copenhagen and Bern, January, 1983

T.C.Bøg-Hansen and G.A.Spengler

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A NOTE ON THE RECENT DISCUSSION ON DEFINITION OF THE TERM "LECTIN"

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Recent years brought a rapid increase of our knowledge of lectins. In spite of their widespread occurrence in Nature, their extensive study as model ligand-binding proteins and their frequent use as specific tools in biological research, at present there exists some ambiguity as to the accurate delineation of the term "lectin". The original Boyd's definition (1) is rather broad and at present it seems necessary to re-define the term more specifically. Efforts in this direction were initiated by Goldstein et al. (2) who stressed sugar-binding and cell agglutinating properties as a basis for the definition. Their definition supplemented by several comments was also published by the Nomenclature Committee of IUB in its Newsletter 1981 (3). The comments specify in a more detail the scope of the definition; sugar--binding proteins with a single binding site are not considered as lectins, whereas sugar-specific enzymes, transport proteins and toxins may be classified as lectins, provided they have multiple sugar-binding sites.

The authors of the present paper have suggested a different definition (4), in which emphasis is put on the sugar-binding properties and the lack of enzymatic activity toward carbohydrate structures, whereas the number of sugar-binding sites or agglutination/precipitation activities are not considered. The Nomenclature Committee of IUB has given preference to the Goldstein's et al. definition (5) essentially because they considered the criterion of cell agglutination

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and/or glycoconjugate precipitation as operational and practical to apply; our definition was found too broad and the criterion of absence of enzymatic activity difficult to apply. However, we still believe that the definition should be based solely on the carbohydrate-binding and not on agglutination or precipitation properties and, also, that sugar-specific enzymes and transport proteins (as well as carbohydrate-specific immunoglobulins) should not be considered as lectins, mainly because:

(1) Cell agglutination is a complex phenomenon depending on the properties of the protein, the cells and of the medium used. Thus the selection of agglutinable cells and optimum conditions may be difficult. Moreover, cell agglutination does not represent - at least in a vast majority of cases - the true physiological activity of lectins. This renders the agglutination-criterion both impractical for general use and rather artifactitious.

(2) Exclusion of monovalent carbohydrate-binding proteins is not logical as they may be structurally and functionally closely related to other multivalent proteins or they may form multivalent aggregates.

(3) Carbohydrate-specific enzymes and transport proteins (regardless of the number of their binding sites) constitute distinct groups of proteins with well defined specialized functions; their classification as lectins might lead to confusion.

(4) The definition should reflect the assumed (and in many cases well documented) carbohydrate-recognition functions of lectins in vivo.

Thus, we suggest a modified version of our original definition (4) which is based on the above considerations:

Lectins are proteins of non-immunoglobulin nature capable of specific recognition and reversible binding to carbohydrate moieties of complex carbohydrates without altering covalent structure of any of the recognized glycosyl ligands.

Comments:

1. A lectin molecule contains one or more sugar-binding sites; lectins may (but do not have to) agglutinate cells or precipitate glycoconjugates as a consequence of binding to their glycosyl moieties.

2. Lectins may possess various biological activities - they can be toxins, hormones etc.; they can be enzymes activity of which is not directed towards sugar molecules.

3. Lectins will usually bind sugars (i.e. low-molecular--weight carbohydrates) which specifically inhibit their interaction with soluble or cell-bound glycosylated macromolecules.

4. Lectins may occur in any type of organisms; they may be soluble or membrane-bound, may be glycoproteins or other posttranslationally modified protein derivatives.

5. Carbohydrate-specific immunoglobulins and any carbohydrate-specific enzymes are not to be classified as lectins. Also carbohydrate-binding proteins complexing only with free sugars but not with complex carbohydrates (some transport proteins, chemotaxis receptors, repressors of operons of carbohydrate metabolism etc.) are not lectins.

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Part I

Biological Effects of Lectins

MECHANISMS OF INDUCTION OF TRANSPLANTATION TOLERANCE IN MICE BY LECTIN TREATMENT

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Administration of some lectins, e.g. Con A or PHA in vivo, results in prolongation of skin or organ graft survival time in such lectin treated recipients (1, 2). Lentil lectin (LCA) was found to be especially efficient in induction of transplantation tolerance in mice (3). In our initial experiments we were able to induce permanent survival of the B10.D2 mouse strain skin grafts in B10.D2(M504) recipients (abbreviated designation M504) after a twenty-dose treatment with LCA. This remarkable effect qualifies LCA as one of the most efficient immunosuppressive (or tolerogenic) substances known so far; a recent study indicates that the efficiency of LCA in rats can be still markedly increased by combination with antithymocyte serum (Harel and Nelken, these proceedings). Therefore, it was of interest to study in more detail the mechanisms underlying these in vivo effects. We tried to answer especially the following questions: (1) What is the immunosuppressive efficiency of LCA in various genetically well defined mouse strain combinations with histocompatibility disparity of different strength? What is the most efficient schedule of lectin treatment? (2) What is the effect of LCA on various functional characteristics of the recipient's lymphocytes? (3) What is the role of active suppressor cell mechanisms in this type of immunological tolerance? (4) What is the fate of LCA in the organism? Has LCA special affinity toward some tissues?

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Materials and methods

LCA and Con A were prepared by affinity chromatography on Sephadex G-100 (4, 5). Two to three months old female mice of various inbred strains were obtained from the animal house of our Institute. Skin grafting was performed by the standard method (6). In adoptive transfer experiments 10^8 spleen cells from the tolerant animals were taken 10 days after the last LCA dose and injected into sublethally irradiated recipients which were subsequently given a skin graft. LCA doses administered i.p. or i.v. were dissolved in 0.5 ml saline, the s.c. doses in 0.1 ml saline. Cytotoxicity of LCA toward spleen cells was estimated in vitro by the ^{51}Cr-release test (7). Stimulation of spleen cell suspensions with LCA was measured by incorporation of ^{3}H-thymidine. Mixed lymphocyte cultures (MLC) consisted usually of 3 components: responder spleen cells, stimulator (either 20 Gy irradiated allogeneic cells or a lectin) and modulator cells. Total culture volume was 0.2 ml; cultures were harvested 48 hours after a 16 h pulse with 36 kBq of ^{3}H thymidine and mean cpm of cell incorporated ^{3}H were measured. Stimulation index was calculated as a ratio of cpm from experimental culture to control unstimulated (or syngeneic cell "stimulated") culture. As a rule, MLC consisted of 10^6 stimulator cells, 5×10^5 - 10^6 responder cells and 5×10^5 modulator cells. Modulator cells were prepared by cultivation of 1.5×10^7/ml spleen cells for 40 hours in the presence of lectins (0.5 - 25 µg/ml) or without lectins (controls). After culture the cells were washed with 0.3 M methyl-α-D-glucopyranoside and pretreated with mitomycin C (50 µg/ml, 30 min, 37°C). Mitomycin treatment was omitted in the case of modulator cells which were subsequently used in the cultures tested for cell mediated cytotoxicity (CMC) development. Cultures for generation of cytotoxic lymphocytes were performed essentially according to Ferguson et al. (8), ^{51}Cr-labelled EL-4 tumor cells were used as target cells. The target vs. effector

cell ratio was 1:20 - 1:2. LCA and bovine serum albumin (BSA) were labelled by ^{131}I using the chloramine T method (9). The rate of elimination of these radiolabelled proteins after injection into mice was estimated by the whole-body radioactivity measurement at various intervals. Distribution of the labelled proteins within the body was followed after killing the animals (24 hours after the injection of the labelled protein), dissection and separate measurement of radioactivity in different organs and tissues.

TABLE 1

Mouse strain combinations used (donor - recipient)

1	2R - B10.A	15	A.TL - A.TH
2	B10.AKM - B10.BR	16	A.TH - A.TL
3	B10.BR - B10.AKM	17	B10.AQR - B10.AKM
4	R103 - M504	18	B10.AKM - B10.AQR
5	B10.D2 - M504	19	B10.AQR - 4R
6	R103 - B10.D2	20	B10 - B10.A
7	B10.D2 - R103	21	B10.HTT - B10.D2
8	B10.A - B10.BR	22	B10.D2 - B10.A
9	B10 - 5R	23	B10.HTT - B10.A
10	B10.A - 4R	24	3R - B10.A
11	4R - B10.A	25	5R - B10.A
12	4R - B10.AQR	26	4R - B10
13	B10.AQR - B10.DBA/1	27	B10.AQR - B10.A
14	B10.DBA/1 - B10.AQR	28	B10.A - B10.AQR

Results

Efficiency of LCA in different strain combinations. 28 different mouse strain combinations with various genetic disparities at the H-2 loci were tested (Table 1).

The efficiency of LCA was clearly dependent on the nature of antigenic disparity between the donor and recepient. In some strain combinations (No. 1-12, Table 1) LCA treatment resulted in permanent survival of the grafts (>100 days) in 80-100% recipients if LCA was administered daily (1 mg, i.p., the doses on the days 1, 3, 5, 7, 9 and 11 were given i.v., in animals bearing the grafts for more than 40 days the lectin was administered alternatively i.p. and s.c. until the rejection of the graft). In all these cases the difference between the donor and recipient is at the H-2DL locus only (No. 1-7) or H-2DL+ some of the H-2J, E, C, and S loci (No. 8-11); only in strain combination No. 12 the donor and recipient differ at the H-2K, J, C, S, DL loci. In other strain combinations involving differences at multiple H-2 loci including H-2K and/or H-2I (No. 13-26) or H-2K only (No. 27, 28) LCA treatment resulted always in marked prolongation of graft survival times as compared to controls (2-4 fold increase of the mean survival time depending on the particular strain combination but permanent survival of skin grafts was achieved only exceptionally. Thus, LCA treatment can be used to overcome the H-2DL antigenic barrier but is less efficient in the case of H-2K or H-2A antigenic disparities.

Cellular mechanisms involved in maintenance of LCA-induced transplantation tolerance. Involvement of active suppressor cell-mediated mechanisms was tested by adoptive transfer experiments. B10.D2 grafts survived permanently in 70% of M504 recipients which were sublethally irradiated and reconstituted with 10^8 spleen cells from M504 recipients bearing B10.D2 grafts as a result of LCA treatment. Only approximately 10% of R103 strain grafts survived permanently in such reconstituted recipients indicating specificity of the state of tolerance but also some degree of nonspecific inhibitory mechanisms involved. Transplantation of third-party test allografts (R103) onto recipients bearing a B10-D2 graft for >100 days

resulted in a rapid rejection of these grafts without affecting the survival of the original B10.D2 grafts.

Effect of LCA on spleen cells in vitro. Mitogenic stimulation of murine spleen cells with LCA was typically dose-dependent (maximum stimulation index approximately 51 at 5 μg/ml in a 5% serum containing medium). The apparent decrease of mitogenic activity at higher concentrations was at least in part due to the cytotoxicity of LCA (approximately 65% of cells were killed after a 24 hours culture in the presence of 0.5 mg/ml LCA; virtually all cells were killed by Con A under similar conditions). Mitogenic response to LCA and Con A of the spleen cells obtained from the M504 mice which were given single 1 mg i.p. dose of LCA (48 hours before testing) was decreased by 93% as compared to untreated controls. There was essentially no mitogenic response in the case of spleen cells from the animals which received a 10-dose LCA treatment (each i.p. dose 1 mg, administered daily, treatment ceased 48 hours before testing). Spleen cells from the B10.A mice which received single 1 mg i.p. dose of LCA manifested a higher proliferative response (1.7 fold as compared to untreated controls) after stimulation with allogeneic B10 cells in MLC. The effects of modulator cells (i.e. spleen cells pre-cultured in the presence of the lectin) on several in vitro characteristics of murine spleen cells were tested. There was a marked difference between the effects of B10.A modulator cells generated in vitro either by LCA or Con A. Whereas addition of Con A-generated modulator cells suppressed the mitogenic response of syngeneic responder cells to LCA stimulation (by 55%), LCA-generated modulator cells enhanced this response (7.5 fold). Similarly, Con A-induced B10.A modulator cells suppressed, whereas LCA-generated modulator cells enhanced the blastogenic response of syngeneic responder cells in MLC toward allogeneic B10 stimulator cells (Hilgert et al., to be published). Both Con A- and LCA-induced modulator cells

suppressed the development of CMC in MLC (by 59% and 65%, respectively, as compared to the cultures containing "modulator" cells pre-cultivated in the absence of any lectin). Similar effect (40-45% suppression of CMC) was observed also when modulator cells generated in vivo were used, i.e. the cells obtained from the spleens of animals pretreated with a single 1 mg LCA dose 2 days before testing or after a 10-dose LCA treatment (ceased 6 days before testing).

Distribution of LCA in vivo and the rate of its elimination. The rate of elimination of ^{131}I-labelled LCA was similar to that of ^{131}I-labelled BSA (approximately 80% of a 1 mg dose administered i.v. was eliminated after 48 hours; LCA administered i.p. or s.c. was eliminated approximately 2-3 times more slowly). Both LCA and BSA were distributed relatively homogeneously throughout the body but LCA possessed increased affinity for spleen, lymph nodes and lung (relative concentration per mg of the tissue was 3.1, 1.5,and 2.9 times higher in these organs, respectively, than the mean concentration in the body), whereas the level of BSA was elevated in blood only.

Discussion

Our results provide at least to some extent the answers to the questions raised in Introduction. First, it seems clear that the immunosuppressive efficiency of LCA depends on apparent strength of histocompatibility barrier between the donor and recipient. The H-2DL antigenic disparity can be relatively easily overcome by LCA treatment, whereas H-2K and H-2A antigens represent stronger histocompatibility barrier; the effects of non-H-2 histocompatibility antigens were not followed in this study. Interestingly, long-term tolerance could be induced only in the B10.D2 - M504 (mutant) strain combination by a limited number of LCA doses. Two in vitro observed

effects may be directly related to the in vivo efficiency of LCA: first, cytotoxicity of LCA at higher concentrations might cause partial depletion of alloreactive cells. Second, the suppressive effect of LCA-generated modulator cells on CMC development might indicate that also in vivo development of alloreactive cytotoxic T cells may be inhibited. This conclusion is also in agreement with our experiments on adoptive transfer of LCA-induced tolerance. This suppressor cell mechanism may be sufficiently efficient for maintenance of B10.D2 graft survival on the M504 recipient after a 20-dose LCA treatment, but the necessity for continuous supply of LCA in other strain combinations tested (with apparently stronger histocompatibility barrier) seems to indicate, that suppressor cells induced in such recipients are not able to "manage" the rejection reaction and that LCA might also block directly some membrane bound or soluble complex carbohydrate molecule(s) taking part in the rejection reaction. The lectin might bind to the histocompatibility antigens of the graft and thus block their recognition. We cannot rule out this effect of LCA in some strain combinations (it might be in agreement with our results on the rate of LCA elimination, which also indicate the necessity for continuous application of LCA to maintain its high (blocking?) level in organism) but the experiments on retransplantation of long-term tolerated B10.D2 grafts onto untreated M504 recipients, transplantation of grafts presoaked in LCA solution or transplantation of grafts from LCA pretreated donors did not reveal apparently any modification of normal immunogenicity of such "LCA-pretreated" grafts (I. Hilgert, unpublished results). Increased affinity of LCA for spleen and lymph nodes (and lung) may support rather the notion that the recipient's immune system is the prime target of LCA action.

In summary, induction of suppressor cells inhibiting graft rejection reaction and direct cytotoxicity seem to be involved in immunosuppressive activity of LCA observed in vivo. Signi-

ficance of other possible effects such as direct blocking of some cell surface or soluble molecules by LCA and some kind of antigenic modulation of the graft remains to be evaluated as well as the roles of antigen-specific and non-specific suppressor mechanisms.

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THE EFFECT OF LENTIL LECTIN TREATMENT ON SKIN ALLOGRAFT SURVIVAL IN MICE AND RATS

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It is well known that concanavalin A (Con A) activated lymphoid cells may suppress in vitro primary and secondary plaque-forming cell responses of mouse spleen cells to heterologous red blood cells (1) and are capable of suppressing cell mediated immune responses in vitro (2). It was shown that Con A inhibited the generation of cytotoxic lymphocytes and that this inhibition was critically dependent upon the concentration and time of addition of Con A or Con A activated cells (2). In vivo experiments showed that intraperitoneal injections of Con A markedly prolonged skin allograft survival in mice (3) and heart allograft survival in rats (4). In a recent article Con A was shown to inhibit immunologically specific cytolytic activity, without any toxic effect on target or effector cells and without interfering with ^{51}Cr release once the lytic lesion has been induced. It was suggested that the inhibition of cytolysis was caused by immobilization in the lymphocyte membrane of various protein moieties by the lectin which directly interfered with events at the effector cell surface (5).

Others have found that the inhibitory effect of Con A on cytotoxic effector cells was dependent upon treatment of the target cells with the lectin. When the targets were preincubated with the lectin, or when the lectin was present in the medium, effector cells were able to kill the targets, with a dependence upon the presence of specific histocompatibility antigens on the target cells (6,7). Similar effects were also observed with phytohaemagglutinin (PHA). When injected intraperitoneally

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under appropriate conditions, it effectively inhibited the rejection of skin allografts, even across the H-2 barrier in mice (8,9). In a recent study, lentil lectin was shown to have a similar effect in preventing the rejection of skin allografts in mice (10). It was shown that the immunosuppressive efficiency of lentil lectin depends on the strength of the histocompatibility barrier between donor and recipient in mice. Lentil lectin was able to overcome the H-2DL antigenic disparity much better than the H-2K and H-2A disparity (11).
The present report deals with skin allografting experiments in mice and rats treated with various combinations of lentil lectin and anti thymocyte serum. It was found that such a treatment may induce a state of allograft acceptance in closely related mice and rats. In adoptive transfer experiments, cells from the treated animals were able to prolong skin graft survival in transplanted secondary hosts.

Materials and Methods

Lentil lectin was prepared as described elsewhere (12). In short, 250g of lentils were homogenized in a Sorvall OMNI-mixer and centrifuged. The supernatant was fractionated by addition of solid ammonium sulfate to obtain the material which precipitated between one and two thirds saturation. The precipitate was dialized against Tris buffer (0.05M, pH 8.1), and passed through a column of DEAE cellulose (Whatman DE-52). The lectin appeared in the first protein peak (OD 280 nm) passing unbound through the column. Activity was detected by hemagglutination of rabbit erythrocytes (13). The product was compared with a commercial lentil lectin, obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and showed a similar potency in hemagglutination tests and an identical pattern in acrylamide gel electrophoresis.
Anti rat thymocyte serum (ATS) was prepared in rabbits by the injection of rat thymocytes emulsified in complete Freund's adjuvant at four week intervals. Two weeks after the third immunization the rabbits were bled, the serum separated, pooled and frozen at -20°C in 10ml aliquots until use. The cytotoxic titer against rat thymocytes with guinea-pig

complement was adjusted to 1:64 in the lymphomicrocytotoxicity test employing the eosin staining method (14). Antisera with weaker titers were not used. A single serum pool was used in each series of experiments. Inbred male Lewis (RT-1^l), Fisher (RT-1^l) rats and inbred BALB/c (H-2^d) and C57Bl (H-2^b) mice were used throughout this study.

Results

In a recent report it was shown that lentil lectin was able to induce skin allograft tolerance in mice (10). In our laboratory, treatment with 8-10 daily doses of lentil lectin alone following transplantation, resulted in prolonged skin allograft survival but a state of tolerance was never achieved (15,16). Using two mice strains with strong H-2 differences, BALB/c and C57Bl, the longest mean skin graft survival time in lentil lectin treated recipients was 22.3±3.81 days (Table 1 group 3) compared to 10.6±1.78 (Table 1 group 8) in untreated control mice. In experiments with rats differing in minor histocompatibility antigens (Lewis to Fisher) treatment with lentil lectin increased the mean survival time to up to 25.1±5.33 days (Table 2 group 2) compared to 10.3±1.46 (Table 2 group 6) in the untreated controls. In another series of experiments the syngergistic effect of rabbit anti rat thymocyte serum and lentil lectin was examined. ATS was injected at the time of the operation followed 48 hours later by the injection of lentil lectin and 48 hours thereafter by another injection of ATS and so on. A total of 8 injections was given, 4 of which were ATS and 4 lentil lectin. This combined treatment consistently resulted in prolonged skin graft survival. In mice the skin allografts survived for about 24 days (Table 1 groups 6,7) and in rats for an observation period over 75 days (Table 2 groups 4,5). In passive transfer experiments we found that spleen cells from ATS and lentil lectin treated Fisher rats with an established Lewis skin graft (Table 2 group 5) were able to prolong graft survival time when transferred to untreated syngeneic rats transplanted with Lewis skin grafts. The transplants were performed 24 hours after transfers of $5x10^7$ spleen cells (Fig. 1). The mean survival time of these transplants was

Table 1

Skin allograft survival in mice (C57Bl to BALB/c) treated with anti thymocyte serum and lentil lectin

Group no.	ATS	LCA	No. of mice	Mean survival time ± S.D.
1	-	5mg/kg, 10 i.p. injections every 24 h starting at transplantation time	25	14.2 ± 2.76
2	-	15mg/kg as group 1	18	16.8 ± 2.94
3	-	25mg/kg as group 1	15	22.3 ± 3.81
4	-	50mg/kg as group 1	13	15.1 ± 4.14
5	0.25ml/mouse 8-10 i.p. injections every 24-48 hours	-	15	18.3 ± 3.77
6	0.25ml x 5 i.p. injections; on days 0, 2, 4, 6, 8 after transplantation	25mg/kg x 5 i.p. injections on days 1, 3, 5, 7, 9 after transplantation	20	24.7 ± 5.12
7	0.25ml x 5 i.p. injections as group 6	25mg/kg 5 i.v. injections as group 6	14	23.6 ± 5.74
8	Control: 0.5ml of 0.9% NaCl 10 i.p. injections every 24 h		30	10.6 ± 1.78

Skin grafts (1 cm in diameter) prepared from the back of the donor mice were transplanted onto the backs of the tested recipients. Grafts with at least 50% necrosis were considered to be rejected.
$p < 0.001$ between groups 1,2,3,4,5,6,7 and group 8. No statistically significant difference was found between group 6 and 7.
S.D. = Standard Deviation
ATS = Anti Thymocyte Serum
LCA = Lens Culinaris Agglutinin (lentil lectin)
i.p. = intra peritoneal
i.v. = intra venous

Table 2

Skin allograft survival in rats (Lewis to Fisher) treated with anti thymocyte serum and lentil lectin

Group no.	ATS	LCA	No. of rats	Mean survival time ± S.D.
1	-	5mg/kg 8 i.p. injections on consecutive days	30	22.3 ± 4.72
2	-	5mg/kg 8 i.v. injections on consecutive days	16	25.1 ± 5.33
3	1ml/rat 8 i.p. injections on consecutive days	-	8	18.6 ± 4.94
4	1ml/rat 4 i.p. injections on days 0, 2, 6, 10 after transplantation	5mg/kg 4 i.p. injections on days 1, 4, 8, 12 after transplantation	10 9	32.1 ± 4.17 > 75
5	1ml/rat 4 i.p. injections as group 4	5mg/kg 4 i.v. injections as group 4	22	all grafts > 75
6	Control: 1ml of 0.9% NaCl, 8 i.p. injections on consecutive days		23	10.3 ± 1.46

Skin grafts (1 cm in diameter) prepared from the chest of the donor rats were transplanted to the chest of the recipients. Grafts with at least 50% necrosis were considered to be rejected.
$p < 0.0001$ between groups 1,2,3,4,5 and group 6.
S.D. = Standard Deviation
ATS = Anti Thymocyte Serum
LCA = Lens Culinaris Agglutinin (lentil lectin)
i.p. = intra peritoneal
i.v. = intra venous

22.2±2.50, compared with 15.0±0.82 in the control group in which transferred cells were from untreated and untransplanted Fisher rats. In several other Fisher rats which showed a perfect take of the Lewis skin allograft (Table 2 group 5), a second Lewis skin graft was performed 8-10 weeks after the first graft. The second graft was rejected in all cases and caused the simultaneous rejection of the first allograft 10-15 days after the operation. When rats, which showed a perfect take of the first Lewis skin allograft were transplanted with a second skin graft from an unrelated Sabra rat, the second graft was always rejected within 7-10 days. In these rats, however, the first skin graft was not rejected (Fig. 2).

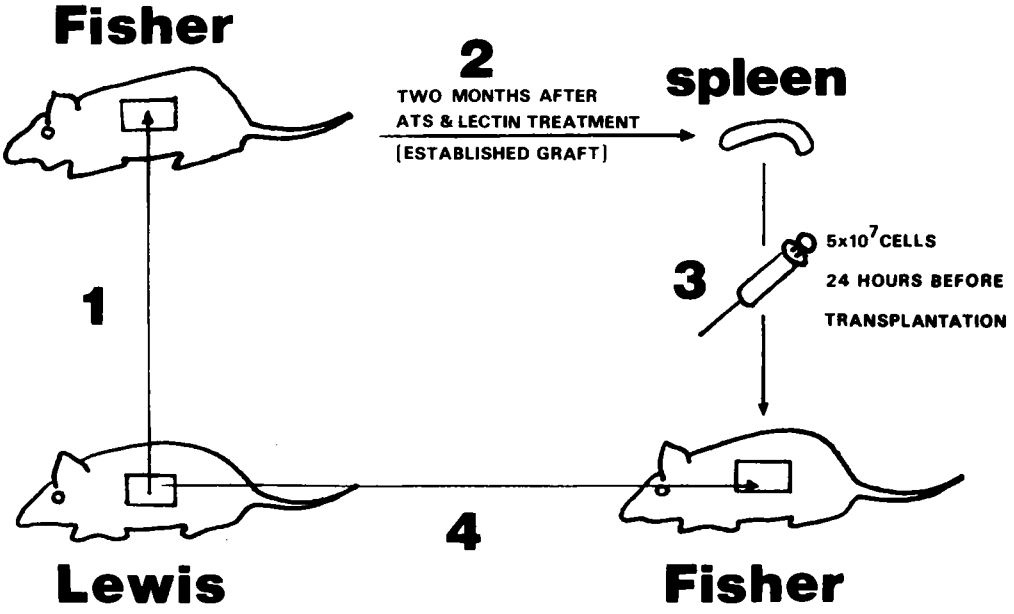

Fig. 1: Passive transfer of $5x10^7$ cells from treated rats with established grafts to newly transplanted rats, causes prolongation of the skin graft survival time.

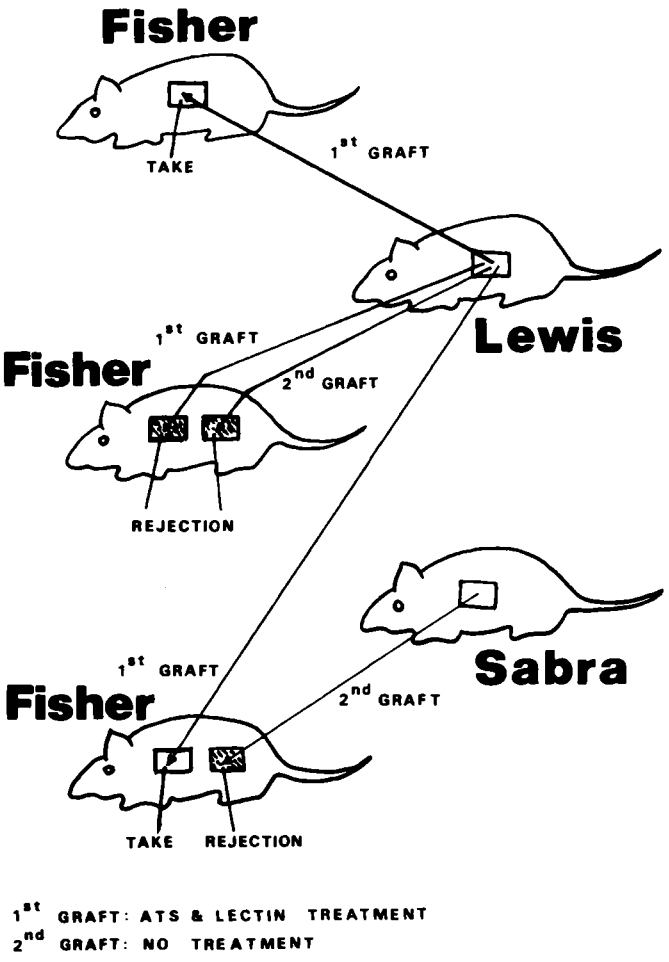

Fig. 2: Transplantation of second set skin grafts to treated rats with an established first graft. When the second graft is from an un unrelated donor (Sabra), it is rejected within 7-10 days, while the first graft survives. When the second graft is the same as the first, both grafts are rejected within 10-15 days.

Discussion

Our results indicate that the combination of anti thymocyte serum and lentil lectin causes a state of acceptance of skin allografts from Fisher to Lewis rats. Cells from the treated rats are able to prolong skin graft survival when transferred to syngeneic recipients transplanted with the same donor type grafts. No real tolerance is achieved, however, since a second allograft from the strain is rejected and seems to cause the rejection of the first otherwise surviving allograft.

In a combination of two mouse strains differing in all the H-2 loci (C57Bl/6 x BALB/c) we were unable to achieve permanent acceptance of the skin allografts by our lentil lectin treatment. These results are in agreement with the recent work of Hilgert, Horejsi et al. who have shown that only in certain genetic combinations lentil lectin treatment could overcome the histocompatibility barrier (11). The same authors suggest that a suppressor cell mechanism is at least partially responsible for the allograft acceptance in the lentil lectin treated mice. We are at present examining this point in rats and mice.

In our present report we were able to reduce the lentil lectin dose and at the same time significantly improve the results of the skin allografts by using a combination of lentil lectin and anti thymocyte serum. This synergistic effect of lentil lectin and anti thymocyte serum points to the eventual clinical feasibility of this treatment in an attempt to improve allograft transplantation.

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POSSIBLE INVOLVEMENT OF LECTINS IN BACTERIA-INDUCED HISTAMINE RELEASE IN INTRINSIC ASTHMA

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A pathogenic role of microorganisms is suspected in the intrinsic asthma disease, since children with intrinsic asthma show a close temporal relationship between exacerbations of symptoms and acute respiratory tract infections (1-3). In our previous study of patients with intrinsic asthma we found that several strains of bacteria caused release of histamine from basophil leukocytes in vitro (3-4). The involvement of histamine release in the disease is supported by the finding of increased urinary excretion of histamine metabolites during acute attacks (5). Bacteria-induced histamine release might therefore contribute to the asthmatic symptoms. The mechanism of bacterial histamine release has been investigated (4, 6). Two different mechanisms seem to exist - an IgE-mediated reaction and a non-immunological mechanism consisting of a direct interaction with the basophil cell surface. Since many bacteria are known to contain surface lectins (7) (see also Wadström and Trust, Eshdat and Sharon, Gilboa-Garber in these proceedings), we studied whether the non-immunological mechanism of the release could be lectin-mediated.

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Materials

Lectins. Con A, Pharmacia AB, Sweden, phytohemagglutinin (PHA), Difco Laboratories, Detroit, USA, poke weed mitogen (PWM) and lens culinaris agglutinin (LCA), IBF, Clichy, France, and wheat germ agglutinin (WGA), peanut lectin (PNA) and ricinus communis agglutinin (RCA) from E.Y. Laboratories, San Mateo, USA.
Carbohydrates. D-(+)-glucose, D-(+)-galactose, D-(+)-mannose and α-methyl-D-mannoside from Sigma, St. Louis, USA, D-(-)-ribose and L-(+)-rhamnose from Merck, Darmstadt, GFR, α-methyl-D-glucoside, Calbiochem AG, Lucerne, Switzerland, and N-acetyl-D-glucosamine, Fluka AG, Switzerland.
Bacteria. Staphylococcus aureus (protein A-free Wood 46) were cultured on solid media and suspended in PBS, pH 7.38 containing 3% formalin. Thereafter the bacteria were washed three times with PBS in the absence of formalin and resuspended in this medium in a final concentration of 120 mg wet weight/ml. The bacteria stock solution was kept frozen at -20°C.

Patients and Methods

Six children with intrinsic asthma (3) and six normal individuals were included in the study.
Preparation of the leukocyte suspension. By venipuncture 10 ml blood samples were collected in plastic tubes containing 0.5 ml 0.20 M EDTA (pH 7.0). 20 ml of 0.9% NaCl was then added to the samples, and the leukocytes were isolated according to the method of Day (8). 10 ml Ficoll-Hypaque solution (12.8 g Ficoll + 21 g Hypaque (sodium diatrizoate) dissolved in water to a final volume of 200 ml). This solution has a density of 1.080 g/ml at 20°C. It was carefully placed below the blood sample. The sample was centrifuged (400 g for 40 min) and the cotton wool-like interphase consisting of monocytes with approx. 2% basophils was mixed with 10 ml Tris-AMC

(Tris (hydroxymethyl) aminomethane 25 mM at pH 7.6, 0.12 M NaCl, 5 mM KCl, 0.6 mM $CaCl_2$, 1.1 mM $MgCl_2$, human serum albumin 0.3 mg/ml). The mixture was centrifuged (107 g for 10 min) and the cell sediment was resuspended in 10 ml Tris-AMC. The suspension was centrifuged (107 g for 10 min) and the supernatant containing thrombocytes was discarded. The cell sediment was finally resuspended in 2.5 ml Tris-AMC.

Histamine release

90 µl leukocyte suspensions containing 2000-8000 basophils were incubated in plastic tubes at 37°C for 40 min with 10 µl Staph. aureus (120 mg wet weight/ml) or 10 µl of the individual lectins in the following concentrations: Con A 10, 30, and 100 µg/ml; PHA 1, 10, 100, and 1000 µg/ml, and 1000 µg/ml of PWM, LCA, WGA, PNA or RCA. Bacteria and lectins were omitted for spontaneous histamine release and for determination of the total histamine content; in the latter case the samples were left at 4°C. Thereafter 3 ml ice-cold Tris-AMC was added to the samples and they were centrifuged at 2000 g for 10 min. Besides released histamine, the supernatant contains also protein and other substances, which interfere with the histamine analysis. It was therefore discarded and the residual histamine was determined in the cell sediment. To the cell sediment was added 400 µl of an OPT mixture (2 mg O-phthaldialdehyde, Fluka, dissolved in 1 ml methanol and 9 ml 0.05 N NaOH). After 4 min 400 µl 0.175 N H_3PO_4 was added and the sample was centrifuged at 3000 g for 10 min. The histamine content in the supernatant was determined spectrofluorometrically in an Aminco-Fluoro-Colorimeter provided with a micro-flow cell. Samples were read at 355/455 nm (excitation/emission). The release of histamine was expressed as a percentage of the total histamine content of the sample. The spontaneous histamine release amounted to 1-10%.

Inhibition studies for examining a lectin-mediated reaction were performed by addition of specific carbohydrates: α-methyl-D-glucoside, α-methyl-D-mannoside, N-acetyl-D-glucosamine, D-(+)-galactose, D-(+)-glucose, D-(+)-mannose, D-(-)-ribose, and L-(+)-rhamnose. The carbohydrates were given individually in concentrations of 0.2 to 20 mg/ml or as a mixture in a final concentration of 0.3 to 2.5 mg of each carbohydrate/ml. They were added to Staph. aureus or to the lectin 10 min before the addition of 80 µl leukocyte suspension.
The presence on the basophils of surface-bound IgE or IgG was shown by histamine release following incubation of the leukocyte suspension with anti-IgE or anti-IgG. Column-immunoabsorbed, no nonsense rabbit-antihuman IgE (9) or IgG (directed against all four subclasses) were used. Cell-bound IgE and possibly also IgG was removed by exposure of the leukocytes to pH 3.8 for 5 min at 4°C (10).

Results and Discussion

Lectin-mediated histamine release from basophil leukocytes was examined in vitro in both patients and normal individuals. Within both groups release of histamine was caused by Con A, PHA, PWM, LCA, WGA, PNA, and RCA. Fig. 1a shows the typical Con A response obtained in all patients and normal individuals. However, this typical Con A response was demonstrated in two different types of patients and normal individuals, i.e. by anti-IgE response cell-bound IgE was only found in one part of the subjects, but not in the other part (Fig. 1b). None of them showed surface IgG. A partial removal of the IgE (Fig. 1b) did not change the Con A response (Fig. 1a). These findings indicate that a lectin-induced histamine release can be independent of the presence of IgE on the basophil cell surface. Another example of a lectin response is given in Fig. 2. The PHA response was low, showing only a 14% release of histamine at the highest concentration of PHA (100 µg/ml). IgE, but not

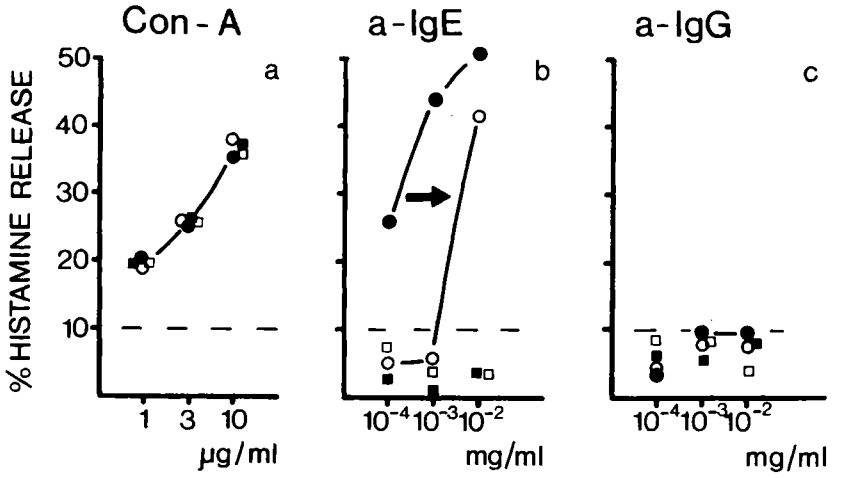

Fig. 1: Histamine release induced by Con A, anti-IgE or anti-IgG in leukocyte suspensions from intrinsic asthma patients or normal individuals. (●) anti-IgE responders, (■) non-anti-IgE responders, (o □) cells treated at pH 3.8 to remove Ig, and (● ■) intact cells.

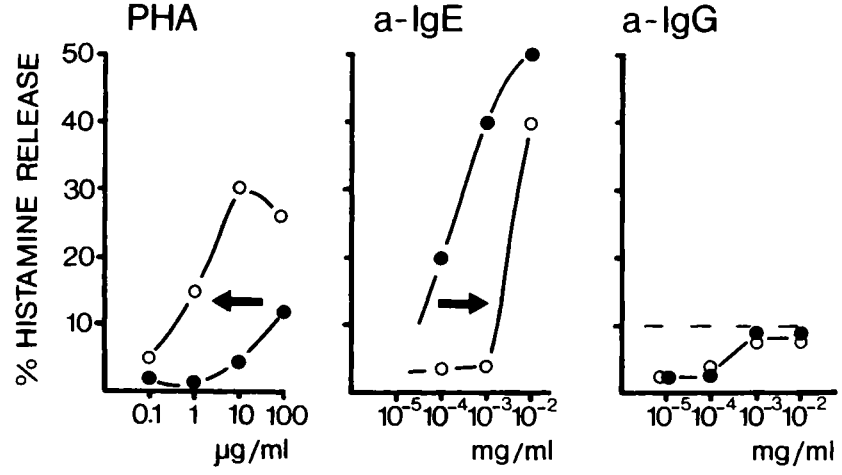

Fig. 2: Histamine release induced by PHA, anti-IgE or anti-IgG in leukocyte suspensions from intrinsic asthma patients or normal individuals. (o) cells treated at pH 3.8 to remove Ig and (●) intact cells.

IgG, was demonstrated on the cell surface. However, a partial removal of the IgE caused a significant increase in the PHA response. By the removal more receptors for PHA binding might be available on the cell surface, and this is possibly the reason for the enhanced response. This pattern was found in both patients and normal individuals. The experiments with Con A and PHA show a lectin-mediated histamine release where lectin bypasses the surface immunoglobulins and interacts directly with the basophil cell membrane leading to release of histamine.
In our previous study of intrinsic asthma we found that bacteria ultrasonicates were able to release histamine by two different mechanisms, an IgE-mediated reaction and a non-immunological mechanism consisting of a direct interaction with the cell membrane (4, 6). The non-immunological mechanism might depend on a lectin-mediated reaction, since many bacteria surfaces are known to contain lectins (see Wadström and Trust, these proceedings), and since lectins by an interaction with carbohydrate moieties on the cell membrane might lead to biological responses such as agglutination, mitogenic activity or release of mediators (see 7, 11, and other chapters in these proceedings). An indirect proof of a lectin-mediated response is the inhibition of the response by specific carbohydrates, which compete with the lectin binding sites on the cell membrane.
The inhibition of the bacteria-induced histamine release by specific carbohydrates was therefore examined in vitro in both patients and normal individuals. Formaldehyde-killed protein A-free Staph. aureus were used. Fig. 3 shows the results of the inhibition studies. A significant decrease in histamine release was obtained when a mixture of eight carbohydrates was included in the medium. In each of the 12 subjects the decrease was significant in the range of 0.3 to 1.2 mg/ml of each carbohydrate ($p < 0.01$ by Student's t-test), and a maximal inhibition of 70 to 90% was obtained. Different patterns of inhibition were obtained in different subjects, and in the day to day experiments, and the inhibition would sometimes be lacking in the same donor. These variations are possibly due to dyna-

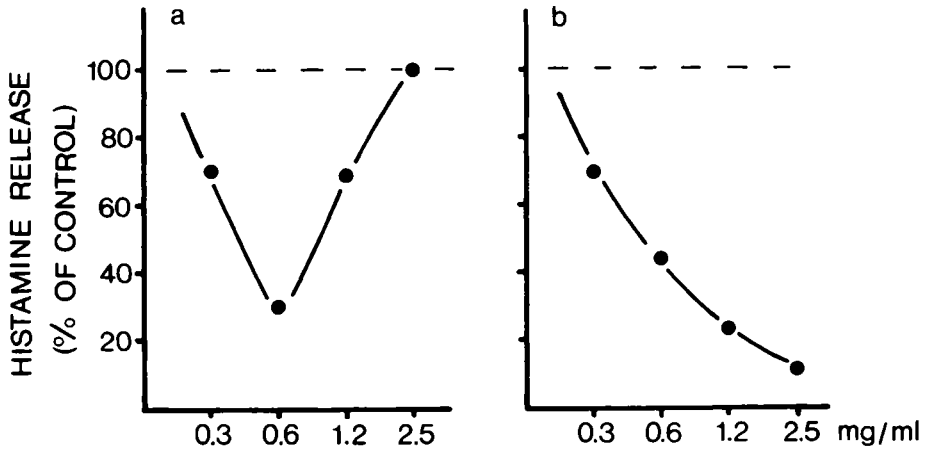

Fig. 3: Representative examples of the two main types of inhibition patterns (a and b) of Staph. aureus-induced histamine release in leukocyte suspensions from intrinsic asthma patients or normal individuals were obtained by the carbohydrate mixture. The mixture included ∝-methyl-D-glucoside, ∝-methyl-D-mannoside, N-acetyl-D-glucosamine, D-(+)-galactose, D-(+)-glucose, D-(+)-mannose, D-(-)-ribose, and L-(+)-rhamnose, each in final concentrations from 0.3 to 2.5 mg/ml. In the absence of carbohydrates (controls) the histamine release varied from 2o to 35%.

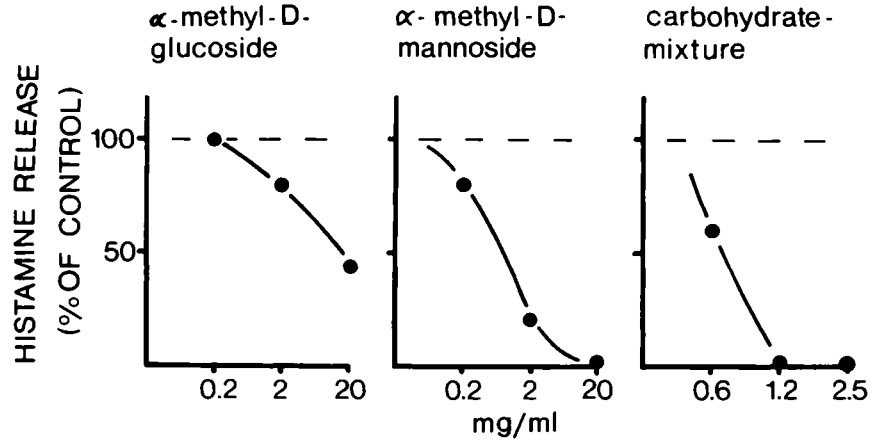

Fig. 4: Characteristic inhibition curves of Con A-induced histamine release in leukocyte suspensions from intrinsic asthma patients and normal individuals by the carbohydrate mixture and by single carbohydrates. The final concentration of the individual carbohydrates is given. In the absence of carbohydrates (controls) the histamine release varied between 2o and 45%.

mic changes in the carbohydrate content of the basophil cell membrane. Fig. 3a and b show the two main types of inhibition, which were found in both patients and normal individuals. In contrast to these experiments performed with the mixture of carbohydrates, no inhibition was obtained when the single carbohydrates were used.

The validity of these inhibition studies for a lectin-mediated reaction was investigated by similar experiments with Con A and PHA. Fig. 4 shows that Con A-induced histamine release was reduced by the carbohydrate mixture and that the inhibition pattern was similar to that obtained with the bacteria (cf. Fig. 3b). However, the release was also inhibited by the individual carbohydrates: α-methyl-D-glucoside and α-methyl-D-mannoside (Fig. 4). Similarly, also the PHA response was found to be reduced by the carbohydrate mixture (Fig. 5), but the effect of individual carbohydrates was not investigated.

In contrast to these lectin experiments the anti-IgE-induced histamine release was not influenced by the individual carbohydrates or by the mixture (data not shown). Nor did the carbo-

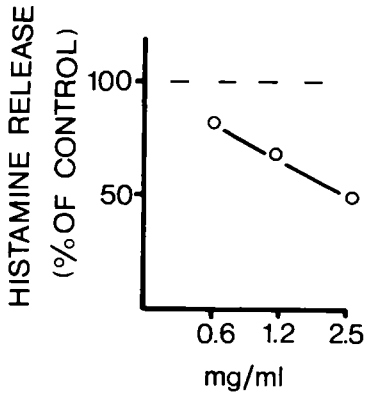

Fig. 5: Inhibition of PHA-induced histamine release in leukocyte suspensions from intrinsic asthma patients and normal individuals by the carbohydrate mixture. In the absence of carbohydrates (controls) the release of histamine varied between 2o and 30%.

hydrates themselves cause any release of histamine. The inhibition studies therefore seem to be an indirect proof of a lectin-mediated reaction.
As mentioned, the carbohydrate mixture inhibited both the bacteria- and the plant lectin-induced histamine release, whereas the single carbohydrate only decreased the plant lectin response. We therefore suggest that the non-immunological mechanism of bacterial histamine release might be caused by a multi-lectin reaction involving several types of lectins on the bacterial membranes reacting with different carbohydrate moieties on the basophil cell surface. On the other hand, the reverse reaction involving basophil surface lectins reacting with bacterial carbohydrates cannot be excluded as a co-operating factor.

Acknowledgements

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T CELL STIMULATING LECTINS ARE MITOGEN INDUCERS BUT NOT MITOGENS

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The discovery that plant lectins, like phytohaemagglutinin or concanavalin A, initiated lymphocyte proliferation (1) has lead to the initial assumption that these lectins were the actual mitogens for lymphocytes. Subsequently, soluble mitogenic factors have been found in culture supernatants of cells stimulated with lectins (2,3). Thus, these endogeneous cell-derived factors, as well as the plant lectins, have been considered to be mitogens. With the observation that lectin-stimulated human peripheral blood lymphocytes secreted an activity that could maintain long-term T cell growth in cultures of human leukemic blood or bone marrow cells (4), it became apparent that the lymphocyte-derived T cell mitogenic activity was actually a growth factor. This mediator was renamed Interleukin 2 (IL-2) in view of its diverse biological activities described earlier, such as lymphocyte mitogenic factor, killer helper factor and helper activity for antibody production. The importance of IL-2 consisted of its ability to support long-term growth of functionally as well as phenotypically normal T cells, leading eventually to the development of clones (5,6,7). In contrast, lectins were found to be an obligatory first signal to activate T cells, but they were not able to cause proliferation by themselves. Rather, lectins were found to cause IL-2 production and

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also the appearance of receptors for IL-2 on T cells (7,8,9). In the present study, we present further evidence from our laboratories that IL-2 provides the second signal more directly responsible for T cell proliferation.

Material and Methods

The production of IL-2 by human mononuclear cells and by the human T cell line HSB-2 as well as the quantitative determination of IL-2 by mouse IL-2 dependent T cell lines have been described earlier (7,10). The monoclonal antibody against activated T cells (anti-Tac) was supplied by Dr. T. A. Waldman (NIH, Bethesda, MD, USA) and has been recently described (11). The monoclonal antibodies against human IL-2 were purified by ammonium sulfate precipitation and subsequent anion exchange chromatography as described (12). Lectins were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and were immobilized with 2,5% glutaraldehyde on plastic tissue culture dishes (Costar, Cambridge, MA, USA).

Results

It has been widely recognized that lectins are mitogenic within a rather narrow dose-range, with higher doses resulting in cell death. Using acridine orange staining of RNA and flow-cytometry we have recently shown (10) two different effects of the lectins (Table 1). Optimal doses of lectins caused a G_0-G_1 shift in the cell cycle, production of IL-2, and subsequent optimal proliferation. However, higher than "optimal" concentrations of the lectins resulted in a block of the cells in G_1 and no proliferation. Nevertheless, production of IL-2 was the greatest at these high concentrations of lectins, suggesting that

TABLE 1

Con A µg/ml	TdR Uptake cpm[a]	IL-2 Units[b]	PHA µg/ml	TdR Uptake cpm	IL-2 Units
0	277	0	0	324	0
1	4,761	4	0.1	2,448	2
10	24,998	6	1.0	36,342	29
50	796	27	10.0	3,074	55
100	728	25	50.0	488	78

a Mean ^{3}H-TdR uptake by triplicate cultures containing 2×10^5 cells pulsed for 4 hr at 44 hr of culture.

b Supernant IL-2 activity of 2×10^6 cells cultured for 48 hr. IL-2 activity was determined on mouse CT6 cells as recently described. (10)

IL-2 was secreted by cells within the G_1 phase of the cell cycle. This correlate between high doses of lectins, G_1 block and increased IL-2 production was confirmed using the mouse EL-4 and the human HSB-2 leukemic T cell lines (10). Contrary to normal resting T cells, these leukemic cells were always in the exponential growth phase, and the lectins were not needed to shift the cells from G_0 to G_1. Therefore, the second effect of the lectins, when high doses were used, was clearly seen: block of the cells in G_1 sufficient to generate supernatant IL-2 activity not seen in the absence of lectins. The correlate between G_1 block and IL-2 production was reproduced in fresh lymphocytes and the cell lines, using the G_1 blocker phorbol myristate acetate. These data suggest that lectins not only are T cell activators, but they also arrest T cells in the G_1 phase of the cell cycle, which eventually leads to accumulation of supernatant IL-2 activity.

In addition to the production of IL-2, the first activation signal mediated by lectins also resulted in the appearance of cell-surface receptors for IL-2. Thus, only lectin-activated

T cells were able to proliferate to IL-2, and they were also able to absorb IL-2 from supernatants (8,9). Thus, the IL-2-receptors appear following the G_0-G_1 shift, and this is similarly dependent on the first activation signal by lectins (10). Binding studies of IL-2 to its IL-2-receptor have not been readily feasible, due to the lack of sufficient highly purified radiolabeled IL-2. However, rabbit antisera and mouse monoclonal antibodies, made by injections of cultured human T cells, could be used to further demonstrate the role of IL-2 and IL-2-receptors (9). Recently, the monoclonal anti-Tac antibody, raised by injections of human cultured T cells, was able to completely block cultured T cells' proliferation to IL-2 at very high dilution of the antibody. The antibody was also able to block the IL-2-receptors on lectin-activated T cells, thus rendering them incapable of absorbing IL-2 activity from IL-2 containing supernatants (Table 2).

TABLE 2

IL-2 Preparation Absorbed on:	TdR Uptake cpm	Comment
RM-1 cells (B cells)	42,700	Control, no absorption
PHA-blasts	25,000	Absorption of IL-2
PHA-blasts, pretreated	42,300	Absorption blocked

Anti-Tac monoclonal inhibits absorption of IL-2 by PHA-blasts. Absorption of IL-2 by PHA-blasts (9), or PHA-blasts pretreated for 2 hr with a 1:2,000 dilution of the anti-Tac. IL-2 assay of the resulting supernatants on secondary cultures of IL-2-dependent cells (TdR uptake).

We have recently described monoclonal anti-human IL-2 antibodies (12), which abrogated the proliferation of IL-2 dependent cultured T cells. Table 3 shows a dose-dependent inhibition of the proliferation. Lectins have no measurable effect

on the proliferation of these IL-2 dependent cells. Thus, the only mitogenic signal for IL-2-dependent cells is IL-2 itself.

TABLE 3

Interleukin 2 units/ml	% INHIBITION OF PROLIFERATION[a] dilution of ascitic fluid antibody[b]			
	1/20	1/40	1/80	1/160
10	55	17	0	0
5	49	35	9	0
2.5	80	52	37	17
1.25	90	70	64	45

[a] IL-2 dependent mouse CT6 cells were cultured in the presence of different concentrations of human IL-2 (= 0% inhibition).

[b] Ascitic fluid anti-human IL-2 antibody were obtained by ammonium sulfate precipitation and were (before adding to the cultures) extensively dialyzed against culture medium (12).

These monoclonal anti IL-2 antibodies were further used to study the role of IL-2 in the lectin induced proliferation of normal resting cells. As shown in Table 4 the addition of monoclonal antibody inhibited also the concanavalin A-induced proliferation. PHA-induced proliferation peripheral blood mononuclear cells was also inhibited by these monoclonal antibodies (data not shown). The monoclonal anti-IL-2 antibodies were not cytotoxic as assayed by dye exclusion. Thus, these data suggest that the IL-2 activity generated by lectin stimulated cells was neutralized by anti-IL-2 antibodies.

Finally we studied whether the T cell stimulating lectins could have an affinity for IL-2. Therefore, we coupled the following lectins to plastic tissue culture plates: concanavalin A, helix pomatia lectin, lentil lectin, leucoagglutinin, phytohaemagglutinin, pokeweed mitogen, soybean lectin and wheat germ lectin. These lectin coated plates were then incubated with 100 units

of IL-2. None of the coupled lectins absorbed IL-2 activity. Thus, lectins that stimulate T cells do not act as a molecular complex with IL-2 to initiate proliferation.

TABLE 4

Hybridoma designation	% inhibition of thymidine uptake[a]		
	1/20[b]	1/180	1/540
B-1	93	71	34
A-1	87	72	68
C-1	77	14	2
D-1	85	59	46
D-2	98	50	56
Control	5	0	0

a % inhibition of maximal cpm (^{3}H-TdR uptake) by 2 x 10^5 human mononuclear cells stimulated with 1 µg/ml concanavalin A.

b dilution of ammonium sulfate precipitated ascitic fluid

Discussion

Several lines of evidence speak in favour of our hypothesis that "mitogenic" lectins act indirectly by inducing hormone-like mitogenic factors or growth factors. It is obvious that lectins are needed to shift T cells from the resting phase (G_0) to the G_1 phase of the cell cycle. Lectins, in fact, act as the first signal that induces the production of the mitogen (= IL-2), as well as the expression of the mitogen receptor (= IL-2-receptor). This hypothesis is also confirmed by the finding that IL-2 dependent cells do most likely not have a resting phase and grow without a lectin stimulus. The growth factor IL-2 alone provides this necessary second signal for proliferation. Therefore, if this mitogenic signal is removed by the addition

of anti-IL-2 antibody, or, if the receptor for IL-2 is blocked by a monoclonal antibody, IL-2 dependent growth will stop. Finally, lectins can block the proliferation of leukemic T cell lines in G_1, and thus lead some of them to produce IL-2.

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MEMBRANE ALTERATIONS AND INDUCTION OF RESPONSIVENESS TO INTERLEUCIN 2 IN LYMPHOCYTES BY LIMA BEAN LECTINS

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Lectins from lima beans have been isolated and characterized by several groups (cf. 1, 2, 3). They specifically agglutinate human blood group A erythrocytes, this agglutination can be inhibited by the addition of the lectin specific sugar N-acetyl-D-galactosamine (1, 2). Lima beans contain several isolectins, which are composed of subunits of MW 31,000. The isolectins of MW 124000 (LIMA 124) and 247000 (LIMA 247) were shown by equilibrium dialysis to possess 2 resp. 4 sugar binding sites (4). Reichert et al. showed in 1973, that a mixture of lima bean isolectins exhibits lymphocyte stimulatory activity (5). When investigating the mitogenicity of 2 purified isolectins towards normal and leukemic human lymphocytes, LIMA 247 could be shown to be a more potent mitogen than LIMA 124 (6). This valency-dependency of the mitogenic properties of the lima bean isolectins could be confirmed in a variety of species, e.g. in bovine, rabbit, rat and mouse lymphocytes (7). It also could be shown, that 2 further isolectins of higher molecular weight (LIMA poly I and LIMA poly II) were able to act as potent mi-

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togens toward human peripheral blood lymphocytes (8). The molecular mechanism of the activation of lymphocytes by lectins is still widely unknown. For lima bean lectin, we could demonstrate binding proteins in the plasma membrane of a human T-cell line (8). The present study shows, that after the binding of lima bean lectin mitogens to lymphocytes, early alterations in the phospholipid metabolism of the cells are induced. Simultaneously to the membrane changes occuring, lectin-treated lymphocytes became responsive to the action of interleukin 2.

Experimental

Lectins. Lima bean isolectins were isolated from bean extracts by fractionated ammonium sulphate precipitation, affinity chromatography on Con A-Sepharose, and gel permeation chromatography as described earlier (3). Concanavalin A (Con A) was prepared from beans (Canavalia ensiformis) by the method of Agrawal and Goldstein (9).

Galactosamine derived Sepharose. A galactosamine derived Sepharose column was prepared as follows: 1 g of D-galactosamine was dissolved in 75 ml water at pH 7.5. To this solution, 15 g CH-Sepharose 4B (PHARMACIA FINE CHEMICALS, Uppsala, Sweden) suspended in water were added. Coupling was done by adding 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide at 0.1 M. The pH of the suspension was adjusted to 5.3 and the mixture was incubated at 4°C for 12 h with gentle shaking.

Lymphocytes. Bovine lymph node lymphocytes were obtained from the local slaughter house and lymphocytes were prepared as described (7). Balb/c mice were from the Institut für Genetik, University of Köln. Mice were sacrificed by cervical dislocation , spleen cell suspensions were depleted of erythrocytes by lysing the red cells (10), and depleted of adherent cells by removing them after carbonyl iron (FLUKA, Buchs, Switzerland) treatment (2 h, 37°C) with the aid of a magnet.

Interleukin 2. (rat T-cell growth factor) was a gift from Dr. M. RETH, Köln, prepared according to Grönvic and Andersson (11) with slight modifications.

Incorporation of Phospholipid Precursors. 10^7 cells of a mixture of spleen and lymph node lymphocytes were incubated in 1 ml Hepes-buffered Dulbecco's modified Eagles medium supplemented with 2.5 mg/ml defatted albumin (12). Precursors of phospholipids were added at the following concentrations: 10 nmol ^{14}C-oleate (specific activity 2 Ci/mol) made by mixing ^{14}C-oleate (AMERSHAM-BUCHLER, Braunschweig, Germany) and oleate (SIGMA, Munic, Germany); and 25 nmol ^{14}C-acetate (specific activity 40 Ci/mol, AMERSHAM-BUCHLER, Braunschweig, Germany). After culturing the cells in the presence of mitogen the lipids were extracted and separated on thin-layer chromatography as described previously (12). Incorporation was measured in total phospholipids and in a fraction containing phosphatidycholine and somé contaminants of phosphatidyl-inositol and phosphatidylserine into which more than 80 % of the phospholipid precursors converted.

Results and Discussion

Lima bean isolectins were purified by fractionated ammonium sulphate precipitation, affinity chromatography, and gel permeation chromatography. Fig. 1 shows the elution diagram of a lima bean lectin purification experiment performed on galactosamine derived Sepharose 4B. As shown in the figure, the isolectins bound to the column could be eluted by a solution of 1 M galactose in PBS. This affinity column yielded very pure lectin preparations, since the purification step involves the interaction of the lectin with its specific sugar. Thus, this technique is superior to the affinity chromatographic purification on Con A-Sepharose which we published previously (3). Using the purification procedure described, an enrichment of the hemagglutinating activity up to 310-fold was achieved. The

hemagglutination titers (2) of the preparations were 70 (LIMA 124), 1460 (LIMA 247), 3100 (LIMA poly II), and 2130 (LIMA poly I). In agreement with the literature, the tetravalent and the polymeric lectins were by far more active agglutinins than the divalent compound (1, 2, 8). The purification steps were controlled by polyacrylamide gel electrophoresis (Fig. 2): In the presence of sodium dodecyl sulphate, the 4 isolectins each exhibited their 31,000 MW subunits as prominent band. Furthermore the isolectins were checked for homogeneity by analytical ultracentrifugation. From the sedimentation patterns of the pure isolectins, the sedimentation coefficients of the substances were determined as 6.26 for LIMA 124, 9.88 for LIMA 247, 11.88 for LIMA poly II, and 13.61 for LIMA poly I. The

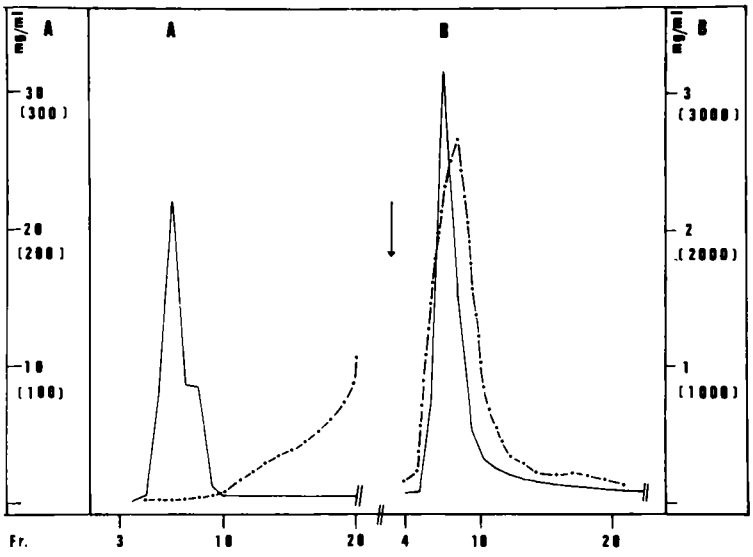

Fig. 1: Purification of lima bean lectins by affinity chromatography on galactosamine derived CH-Sepharose 4B. 800 mg of protein were applied to the column (l = 17.5 cm, d = 2 cm) in phosphate buffered saline (PBS), and the material not attached to the column was eluted (A). Lectins bound to the column were eluted with PBS containing 1 M galactose (B). Solid line: protein content of the eluted fractions (mg/ml). Broken line: hemagglutination titers. The preparation of the column was performed according to Allen and Neuberger (16) with modifications as described in Experimental.

results presented in figures 1 and 2 show that by the purification method employed, 50 mg amounts of the lima bean isolectins exhibiting a high purity can be isolated from 60 g of lima beans in a quick and reproducible manner. The combined method is also applicable for the purification of other galactosamine specific lectins (comp. 3, 16).

We had shown previously that the mitogenicity of the lima bean isolectins towards lymphocytes of a variety of species is dependent from their saccharide binding valency, and that the polymeric isolectins activate macromolecular syntheses to a degree superior to the isolectin LIMA 124 (6-8). These findings

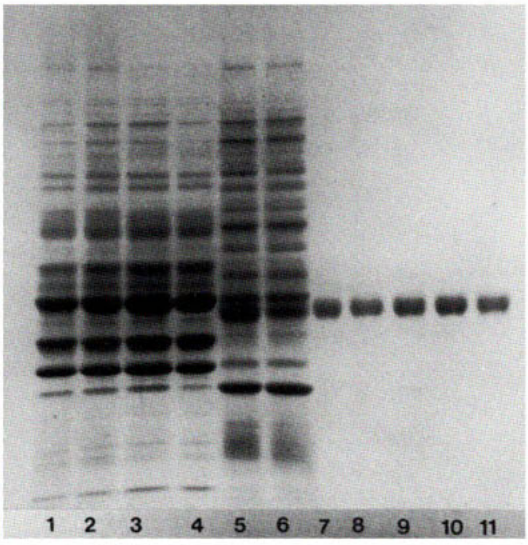

Fig. 2. Purification of the lima bean isolectins as controlled by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS). Gradient slab gels (10-15 % acrylamide) prepared according to Laemmli (17).
Crude lectin extract (lane 1), supernatants (lanes 2-4) and final sediment (lane 5) of the fractionated ammonium sulphate precipitation (cf. ref. 3). Fractions non binding (lane 6) and lectins binding (lane 7) to the galactosamine derived Sepharose 4B column (see Experimental). Isolectins after their separation by gel permeation chromatography on Sephadex G200 superfine (cf. ref. 3): LIMA poly I (lane 8), LIMA poly II (lane 9), LIMA 247 (lane 10), and LIMA 124 (lane 11).

were confirmed in our experiments measuring the early alterations in the phospholipid metabolism of lectin stimulated lymphocytes. Since relatively large amounts of cells were necessary for these assays, bovine lymph node lymphocytes were used. In the assays, the effects of divalent LIMA 124 and tetravalent LIMA 247 were compared; according to gel electrophoresis LIMA 247 contained some aggregated polymeric material (cf. 1, 3, 7). LIMA 124 and LIMA 247 both stimulated the incorporation of ^{14}C-oleate into phosphatidyl choline starting at concentrations around 10 µg/ml, the stimulation optimum was around 150 µg/ml for LIMA 124, and around 50-100 µg/ml for LIMA 247. For concanavalin A, the optimum response was found at concentrations

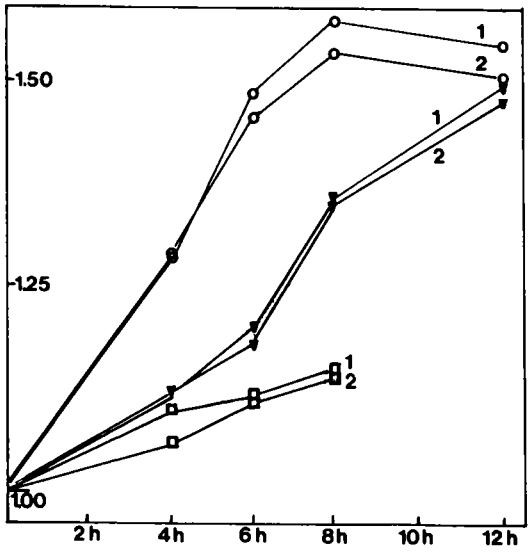

Fig. 3. Kinetics of incorporation of ^{14}C-oleate into membrane phosphatidyl choline (1) and into membrane phospholipids (2) in lymph node lymphocytes after various times of incubation of lymphocyte cultures with 1.6 µg/ml concanavalin A (o), 100 µg/ml LIMA 247 (▾), and 150 µg/ml LIMA 124 (▫). Results are means of triplicate or quadruplicate assays. Controls without addtion of lectins: 4065 cpm for phosphatidyl choline, and 5037 cpm for membrane phospholipids. Ordinate: stimulation indices = cpm of mitogen stimulated cultures/cpm of control cultures. Abscissa: time of incubation.

around 1 µg/ml, which is in agreement with our results published for mouse lymphocytes (12). The summarizing fig. 3 shows the kinetics of oleate incorporation induced by the lectins. As seen from the figure, both lima bean isolectins stimulated the incorporation of ^{14}C-oleate into membrane phospholipids and membrane phosphatidylcholine already 4 hours after their addition to the cultures. LIMA 247 exhibited a much more pronounced effect than the divalent isolectin, and after 8-12 h, the effect of LIMA 247 became comparable to concanavalin A; the incorporation at this late time, however, is partly due to de novo net synthesis of phospholipids due to the mitogenic activation of the cells. Our results show that the membrane events induced by the lima bean isolectins are dependent from their valency,

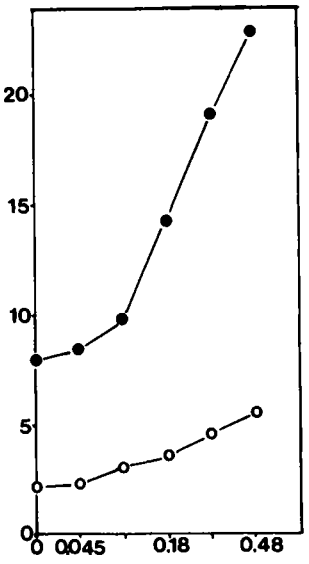

Fig. 4. Induction of responsiveness to interleukin 2 in lymphocytes by LIMA 124. Balb/c mouse lymphocytes were preincubated at a cell concentration of 1.2 x 10^7/ml with 0.4 U/ml neuraminidase (30 min, 37°C). After washing, LIMA 124 was added to the cell cultures at different concentrations. Cultures were performed in COSTAR microtiter plates at 3 x 10^6 cells/ml for 42 h; 18 h before harvesting, 0.2 µCi ^{3}H-thymidine were added to the wells. (o) Cultures performed without interleukin 2. (●) Cultures performed in the presence of 10 % interleukin 2 containing supernatant (see Experimental). Ordinate: ^{3}H-thymidine incorporation (cpm x 10^{-3}). Abscissa: lectin concentration (mg/ml).

as shown before for their mitogenic activity (6-8). A similar valency dependent stimulation of phospholipid metabolism by lima bean isolectins could also be shown, in preliminary assays, for human, rat and mouse lymphocytes (unpublished). These lectin induced changes, which we also could demonstrate for B-lymphocytes using the B-lymphocyte mitogens lipoprotein and lipopeptide (12), involve an enhanced uptake of unsaturated fatty acids into membrane phospholipids. The induced fluidization of the plasma membrane brings about a modulation of the activities of membrane associated enzymes, which could lead ultimately to lymphocyte proliferation and differentiation (13, 14, 15).

Simultaneously to the induction of membrane events, the divalent lima bean isolectin, which is only marginally mitogenic towards mouse lymphocytes, was able to induce responsiveness to interleukin 2 in Balb/c mouse lymphocytes. The effect could be further enhanced by pretreatment of the lymphocytes with neuraminidase. Fig. 4 shows, that in the presence of 0.2 - 0.4 mg/ml LIMA 124, ^{3}H-thymidine incorporation could be enhanced 4-5 fold by the addition of 10 % of rat interleukin 2 supernatant to the cultures. Similar assays employing LIMA 124 have been used by us to test for interleukin from different sources without the aid of interleukin-dependent cell lines or mitogenic concanavalin A (cf. ref. 19).

The results presented here might suggest that LIMA 124, which exhibits only a marginal mitogenicity towards mouse lymphocytes (7), induces membrane alterations in T-lymphocytes followed by the functional appearance of the receptor for interleukin 2 proposed by several groups (cf. ref. 19-21). On the other hand, the divalent LIMA 124 might be unable to induce, by itself, the production of the interleukins necessary for the mitogenic activation of the T-lymphocytes (21). A further investigation of these hypotheses is in progress in our laboratories.

Acknowledgement
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T LYMPHOCYTE-MEDIATED CYTOLYSIS

IV. How do Mitogenic and Non-Mitogenic Lectins Mediate Lymphocyte - Target Interactions?

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Cytotoxic T lymphocytes (CTL) can lyse target cells (TC) nonspecifically in the presence of certain plant lectins, notably Con A and PHA. Lectin-dependent cytotoxicity (LDCC) has been attributed to CTL-TC interactions mediated by intercellular 'bridging' of multivalent, carbohydrate-specific lectins, followed by 'activation' of the CTL by the lectin (1-3) (see scheme)

$$CTL + TC + L \rightarrow CTL^* - L - TC \rightarrow Lysis + TC$$

(L, lectin; CTL*, activated CTL)

Because the mechanism of LDCC is highly relevant to specific, CTL-mediated lysis, we investigated in detail how lectins mediate CTL-TC interactions. In previous communications of this series (4-6), we presented evidence that the lectin Concanavalin A does not act simply to 'bridge' CTL and TC, nor does it 'activate' the CTL. Instead, we proposed that lectin modify or cluster cell-surface determinants of the TC, rendering them nonspecifically recognizable through CTL surface receptors other than lectin-binding sites. The present paper delineates possible mechanisms whereby some mitogenic and non-mitogenic lectins mediate CTL-TC interaction in LDCC.

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Materials and Methods

These have been described in detail previously (4,5). As CTL we used either Con A-activated lymphoblasts or alloimmune peritoneal exudate CTL. As TC we used lymphoma EL4 of C57BL/6 or P815 of DBA/2 mice. CTL-TC binding and lysis were assessed by the conjugation and ^{51}Cr-release assays, respectively.

Results and Discussion

The observation that in the presence of certain plant lectins CTL can lyse virtually any TC, including syngeneic and even antologous cells (7-9), is in sharp contrast to experiments showing selective inactivation of only B anti-C CTL following their interaction with A anti-B CTL and, likewise, lack of simultaneous, mutual lysis when A anti-B CTL are confronted with B anti-A CTL (see refs. 10, 11 and Fig. 1).

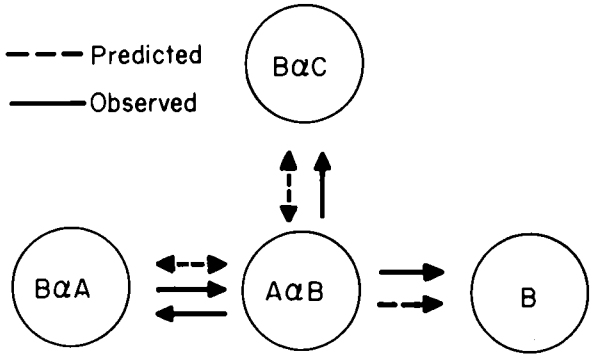

Figure 1.
Interaction of CTL and TC which are themselves CTL.

These killer- anti-killer experiments have indicated that TC binding through the CTL receptor is a prerequisite for subsequent lysis, suggesting that 'passive' binding of a CTL to its TC, although required, is not sufficient for affecting lysis of the latter. The previous interpretations of the mode of action of lectins in LDCC, (1-3) suggesting a 'dual' role of lectin stem primarily from the finding that only T cell mitogenic lectins such as Con A PHA and LCA can mediate LDCC while non-mitogenic lectins such as PNA and WGA

do not (2). Although it has been assumed that non-mitogenic lectins can mediate 'effective' CTL-TC binding not leading to lysis, this assumption has never been substantiated by a CTL-TC conjugation assay, for example. In fact, direct examination of the CTL-TC conjugating ability of mitogenic (LDCC-supportive) and non-mitogenic (LDCC-nonsupportive) lectins revealed that while effective CTL-TC conjugation can be easily demonstrated with mitogenic lectins such as Con A, PHA and LCA, virtually no conjugates could be detected with non-mitogenic lectins (Table 1). These results provide a new basis for the lack of LDCC activity of non-mitogenic lectins such as PNA, WGA and SBA. Hence previous explanations of the dual role of Con A in LDCC

Target cells	Immune CTL	
	BALB/C anti EL4	C57BL/6 anti-P815
	(Number of conjugated/0.5 microliter	
EL4	60	2
P815	4	87
Con A·EL4	-	86
Con A·P815	60	-
PNA·EL4	-	5 (2)
PNA·P815	1 (3)	-
SBA·EL4	-	23 (2)
SBA·P815	3 (6)	-
WGA·EL4	-	2 (3)
WGA·P815	2 (3)	-

Table 1. CTL-TC conjugation by mitogenic and non-mitogenic lectins

BALB/C anti-EL4 or C57BL/6 anti-P815 alloimmune CTL were mixed with lectin-treated or untreated EL4 or P815 TC ($1x10^6$ cells each), centrifuged 170 xg, 10 min at room temperature to affect conjugate formation. Pellets were resuspended vigorously with a Pasteur pipette and the number of conjugates per 0.5 microliters was determined microscopically (see ref. 12). Numbers in parentheses show number of conjugates in the absence of lectins.

(1-3,13) must be revised as it rests on the assumption that both mitogenic and non-mitogenic lectins are equally effective in mediated CTL-TC conjugation.

Binding (even temporary) of CTL to TC is an essential, early step in a multistep process, ultimately leading to TC lysis (see ref. 12 for review). Although morphologically indistinguishable conjugates have been detected in