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REGULATION OF THE IMMUNE RESPONSE
~~BY PROTEASE INHIBITORS~~

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ROLE OF PROTEASE INHIBITORS IN THE REGULATION OF THE IMMUNE RESPONSE

By

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ABSTRACT

ROLE OF PROTEASE INHIBITORS IN THE REGULATION
OF THE IMMUNE RESPONSE

By

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For a long time cell biologists have proposed that proteolytic enzymes play a role in cell proliferation and lymphocyte differentiation. Protease inhibitors [P.I.] on the other hand inhibit these proteolytic enzymes thereby abrogating their functions. Two protease inhibitors alpha-1-antitrypsin [α_1-AT] and Trasylol were examined for their role in the regulation of the immune response. An in vitro modified plaque forming cell [PFC] assay was used to measure the immune response.

α_1-AT was found to have a regulatory effect on the immune response. In concentrations of 100 to 1000 µg/ml of murine spleen cell culture, α_1-AT significantly suppressed the anti-SRBC PFC response. In vivo studies confirmed these findings. Results indicated that this protein does not bind to antigen, and it does not lose its biologic activity under normal cell culture conditions. α_1-AT was found to be homogeneous, when examined by isoelectric focusing and by disc gel electrophoresis. α_1-AT suppressed antigen-dependent B-lymphocyte differentiation without affecting adherent or T-cells.

Since one of the unique properties of Trasylol is its protease-inhibiting capacity similar to that of α_1-AT, this kallikrein inactivator

was tested as a model of protease inhibitor for further determining various parameters of immuno-regulation brought about by protease inhibitor. In concentrations of 100 to 2000 kellikrein inactivating units [KIU] per ml of culture, this inhibitor suppressed both the primary and secondary PFC response. Suppression by Trasylol was not antigen-specific. This was further verified by the demonstration that Trasylol suppressed *in vitro* PFC response of spleen cells against dextran. Suppression by Trasylol was not due to depletion effect on the antigen. Inhibitory capacity of Trasylol was reversible and the degree of suppression was dependent on the time at which trasylol was added to the cultures. Trasylol added to antigen-stimulated cultures up to 48 h after initiation of cultures was immuno-suppressive whereas at 72 h after initiation or later it did not suppress. Pretreatment of spleen cells with this inhibitor, for 6 h before exposure to the antigen did not affect the immune response. When pre-incubated with trypsin, the suppressive activity of Trasylol was abrogated. Similar to our previous observations with α_1-AT, Trasylol did not appear to affect T-cells or adherent cells, but it suppressed the B-lymphocyte differentiation suggesting that Trasylol has an immunoregulatory function.

By the use of incorporation of radiolabeled thymidine ([^{3}H]-thymidine) into mouse spleen cells and plasmacytoma [MOPC-21], the inhibitory effect of Trasylol on mitogen-induced lymphocyte triggering was also studied *in vitro*. DNA synthesis was effectively inhibited by 250 to 1000 KIU of Trasylol when response was induced by lipopolysaccharide [LPS] of *Escherichia coli*. The inhibitory effect of Trasylol

was reversible. On the contrary, DNA synthesis of spleen cells was not inhibited by the inhibitor when the cells were stimulated with 1 μg of concanavalin-A [Con-A] per culture. DNA synthesis and growth rate of plasmacytoma, a B-cell tumor, was also reversibly inhibited by Trasylol. Taken together these results suggest that the target of inhibitory action of protease inhibitors such as α_1-AT and Trasylol was the B-lymphocyte. Fluorescent labeling studies have also supported these findings since B-lymphocytes and plasmacytoma display intense binding of labeled protease inhibitor [both Trasylol and α_1-AT] compared to low levels found on T cells or lymphoblastoid cell line [S.49.1, T-cell tumor]. The functional significance of these protease inhibitors as regulators of lymphocyte differentiation and tumor growth is discussed.

DEDICATED TO

MY PARENTS

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INTRODUCTION

The role of proteolytic enzymes in cellular functions has been expanded in recent years. Proteases have been found on the surface and in the medium of cultured cells and it has been suggested that a proteolytic mechanism may be of importance in cell proliferation and lymphocyte differentiation. Protease inhibitors, on the other hand, are known to inhibit or inactivate proteolytic enzymes thereby abrogating their function. Furthermore they also inhibit proteases isolated from lymphocytes. The major objective of this research was to investigate the role of alpha-1-antitrypsin [α_1-AT], a major protease inhibitor present in the normal serum, in the regulation of the immune response and to determine the type of lymphoid cell population regulated by the protease inhibitor. Trasylol is a protease inhibitor similar to α_1-AT, and was used as a model for further determining various parameters of immunoregulation brought about by protease inhibitor.

Literature on the protease inhibitors, α_1-AT and Trasylol is presented with respect to the biochemistry, genetics, mechanism of protease inhibition and the inhibition spectrum, and their role in pathogenesis and the immune response is also discussed. Finally the role of α_1-AT and Trasylol in the regulation of the immune response is examined with emphasis on specific cell populations regulated by the protease inhibitor.

In these studies we proposed that alpha-1-antitrypsin, a constituent of normal human serum, has an immunoregulatory function based on demonstration of a non-cytotoxic immuno-suppressive effect on the primary antibody response both in vitro and in vivo by this protease inhibitor. Furthermore, it was observed that α_1-AT suppressed antigen-dependent B-lymphocyte differentiation without affect on adherent or T-cells. Results of these experimental approaches are presented in the first of three manuscripts [published in Nature, 274:589 (1978)]. The second article [submitted for publication] extend this concept by examining the effect of polyvalent enzyme inhibitor Trasylol on the primary and secondary immune response to both T-dependent and T-independent antigens. This investigation is the first study in which Trasylol has been examined for its effect on the immune response. Further experiments have been carried out to investigate if immunoregulation by Trasylol is due to its protease inhibiting capacity and also to determine its mode of action. Finally, the third article [submitted for publication] focuses on the role of Trasylol in its inhibition of lymphocyte stimulation by mitogens and gives evidence that this action is exerted only on B-lymphocytes of spleen. Fluorescent microscopy was used to verify that protease inhibitor binding was more selective for B-lymphocytes.

LITERATURE REVIEW

Immuno regulation, a fundamental and ubiquitous mechanism, can be brought about by certain normal serum proteins. Feedback inhibition of the immune response by antibodies represents a specific system of regulation (1). Immune-regulatory factors which nonspecifically inhibit an immune response are subjects of great interest but remain poorly understood.

The bulk of experimental evidence to date suggests that a number of proteins present in normal human serum can serve as immuno-suppressors or as immuno-enhancers. Recently α-globulin rich fraction of Cohn Fraction IV, designated IRA (immune-regulatory alpha globulin) has been implicated in suppressing the in vitro antibody response of spleen cells to sheep red cells without cytotoxicity (2). Results from the studies of Chase (3) also indicate that α_2-macroglobulin, one of the protease inhibitors [P.I.] present in normal serum limits the human lymphocyte response to phytohemagglutinin [PHA-P] and Concanavalin-A [Con-A] as measured by ^{3}H-thymidine incorporation. A similar role for α_1-antitrypsin, a protease inhibitor and a constituent of the normal serum can be postulated to be involved in the regulation of the immune response.

The role of proteolytic enzymes in cellular functions has been expanded in recent years. Proteases have been found on the surface and in the medium of cultured cells (4). They have been implicated in altering the electrophoretic mobility of lymphocytes (5), in lymphocyte

migration in vivo (6), and even in lymphocyte blast transformation (7).

Protease inhibitors [P.I.] on the other hand, have a function to inhibit proteolytic enzymes produced by leukocyte granules (8). Furthermore, they also inhibit proteases produced by certain bacteria during their infection (9). More recently protease inhibitor like N-α-tosyl-L-lysyl chloromethyl ketone [TLCK] has been shown to affect an intracellular protease thought to be responsible for lymphocyte blastogenesis (10).

The function of α_1-antitrypsin [a protease inhibitor] in the serum is unknown, nor is it known why there is an increase in serum of this proteinase inhibitor in certain pathological conditions (11). One intriguing possibility is that α_1-antitrypsin has immuno-regulatory properties, which are important for the exemption of intracellular pathogens and even tumor cells from immuno-logical attack.

Immuno-suppression by protease inhibitor, α_1-antitrypsin, as has been demonstrated by us [published results] may lead to further understanding of the severe deficiency diseases of the immune system, an association between protease inhibitor and immune deviation and finally the relationship between immune deviation and disease.

I. Protease Inhibitors

The earliest observation concerning the anti-tryptic activity of serum date back to the end of the last century (12) and the first attempts to concentrate and isolate protease inhibitors [P.I.] were reported by Landsteiner in 1900 (13). However, it has been only during

the last 25 years, that nearly six P.I.'s of human plasma have been isolated and chemically and biologically characterized.

Protease inhibitors are found not only in plasma, but also in organs and tissues sensitive to proteolysis, i.e., the lungs, mucous membranes of the nasal respiratory and G.I. tract (14).

Although a number of P.I.'s are already well characterized, only α_1-antitrypsin [α_1-AT] and α_2-macroglobulin [α_2-M] have turned out to be important with regard to both their concentration in the plasma and specificity; the other inhibitors are only present in trace amounts. Heimburger (14) lists six different protease inhibitors well characterized in human serum (Table 1).

Table 1.* Protease Inhibitors in Human Serum

		Normals (mg/dl)
α_1-Antitrypsin	α_1-AT	180-280
α_1-Antichymotrypsin	α_1-X	30-60
Inter-α-trypsin inhibitor	IαI	20-70
Antithrombin III	AT III	22-39
Cl-Inactivator	Cl INA	15-35
α_2-Macroglobulin	α_2-M	150-350 males
		175-420 females

*Data taken from Table 1 of Reference #14.

A. Alpha$_1$-Antitrypsin

Since the first successful isolation of α_1-AT by Moll, Sudden and Brown in 1958 (15) nothing much was done on α_1-AT until 1964, when Laurell and Erickson (16) described α_1-AT-deficiency in man and established an association between homozygous α_1-AT deficiency and obstructive emphysema of lungs. Since then a number of phenotypes of α_1-AT deficiency have been reported in the literature. It has been observed that the frequency of chronic pulmonary emphysema is significantly higher in subjects with homozygous deficiencies of α_1-AT than in the general population (11).

α_1-antitrypsin [α_1-AT] has been identified as an alpha-1-globulin. Enzymes shown to be affected by its inhibitory properties include trypsin, chymotrypsin, pancreatic elastiase, skin collagenase, renin, urokinase, Hageman factor cofactor and the neutral proteases of polymorphonuclear leukocytes (17,22). These enzymes include an elastase, a collagenase and a non-specific protease capable of digesting vascular basement membranes (23,24). α_1-AT can also inhibit the acid protease present in alveolear macrophages (25). Inhibition of kallikrein is negligible (26).

α_1-Antitrypsin can be found in a number of body fluids such as tears, perilymph, lymph, saliva, colostrum, mother's milk, duodenal fluid, gall bladder bile, synovial fluid, cervical mucus, semen and amniotic fluid (17,27-31). It is also found in platelets and in megakaryocytes (32). In the serum, exogenous α_1-AT has a half life of only about a week, most of the loss being by catabolization in the liver (33).

Biochemical Characterization: Although the α_1-AT isolated from deficient patients can be distinguished electrophoretically from that of normal persons, immunologically and functionally [i.e., as an inhibitor of trypsin or elastase in vitro] it is identical. This glycoprotein has a molecular weight ranging from 49,500 to 54,000, consists of a single polypeptide chain [389 amino acids with the predominance of aspartate, glutamate, leucine and lysine]. The carbohydrate content [nearly 12%] consists of four side chains of two different types [containing hexose, glucosamine and sialic acid] (34). In severely deficient persons it has less N-acetyl-glucosamine, mannose, galactose and sialic acid [N-acetyl-neuraminic acid] than that from normal persons (35). The isoelectric point is in the pH range of 4 to 6.

Determination and Isolation of α_1-AT: α_1-Antitrypsin is usually estimated by radial immunodiffusion (36) or by measuring the trypsin inhibiting capacity of serum. In the latter test the residual activity of trypsin is measured after the enzyme had been incubated for a fixed period of time with a dilution of the serum to be tested (37). More recently Delforge (38) did crossed immunoelectrophoresis of α_1-AT using thin layer isoelectrifocusing [TLIEF] in polyacrylamide as the first dimension. This system provided increased resolution of the patterns associated with the polymorphic expression of α_1-antitrypsin.

Isolation of α_1-AT from serum is a difficult procedure and the results are not always satisfactory. Kueppers (36) isolated apparently pure α_1-AT by electrophoresis on a Pevikon block with subsequent filtration on a Sephadex G-200 column, but one of the steps included removal

of sialic acid by neuraminidase. Such desialylated α_1-AT preparation preserved its antigenic properties and after labeling with ^{125}I behaved *in vivo* as a native protein (39). Musiani and Tomasi, Jr. (34) used affinity chromatography employing an antiserum which had been depleted of α_1-AT antibodies. Their final preparation was homogeneous by immunological and physiochemical criteria. Hao *et al.* (40) fractionated human plasma by using polyethylene glycol and obtained purified [>95%] α_1-AT with a yield of nearly 14 percent.

Genetic Aspects of α_1-AT: Functionally and immologically the α_1-AT proteins are identical. Only by electrophoresis on starch gel in discontinuous acid buffers and by isoelectric focusing [IEF] can variants be detected with certainty. A system of labeling based on letters of the alphabet has been adopted, the electrophoretically slowest being denominated Z, the usual M, and the faster F. S, the second most common type, falls between M and Z. The system itself is titled "Pi" for protease inhibitor.

Although reports of at least 26 different Pi alleles have appeared in the literature since the system of letters was agreed upon, a number of the alleles have apparently been found in only one individual or one family. All available data support the hypothesis of codominant alleles, that is, each allele controls the production of one kind of α_1-AT molecule uninfluenced by the corresponding allele on the other member of the chromosome pair. No more than two sets of bands have ever been found in a single subject's pattern (41,42) although mosiacism is theoretically possible. Presumably, these are at a single locus, but the specific

chromosome carrying the Pi genes has yet to be determined. Evidence for linkage with the Gm gene, which codes for the constant part of the heavy chain of IgG, is rather good and favors chromosome 2 (43,44). There does not appear to be good evidence for any other linkage (42,44).

Pi^M is by far the most common allele with a frequency of at least 0.87 (45). Other alleles include Pi^S, Pi^Z, PiMZ, PiZZ and PiSZ (45). A few cases have been discovered in which levels of circulating α_1-AT are virtually undetectable and no electrophoretic pattern at all is obtainable (46,47). Lack of display of characteristic PAS-positive globules in the liver strongly suggests that deletion of the Pi gene has occurred, and the allele is denominated Pi^{null} or Pi^-.

M allele has been further divided into a number of subtypes. Johnson (48) reported M_{lamb} and $M_{Baldwin}$ subtypes with acid starch gel electrophoresis. Kueppers (49) reported a heterozygous subtype Pi MM_1. Others (50,51) reported M_1M_2 subtypes. Genz et al. (52) further divided the subtypes into the homozygous types Ma, Mb, and Mc and the heterozygotes into Mab, Mac, and Mbc. There is considerable overlap in the terminologies of these subtypes and a standard nomenclature has yet to be determined by workers in this field.

Mechanism of Inhibition by α_1-AT: The serine proteases that are inhibited by α_1-AT appear to cleave peptides by attaching the active catalytic site of the enzyme to one of two particular locations in the amino acid chain of the peptide (18). They probably combine with the α_1-AT molecule in the same fashion (18). Since chemical modification of the lysine residues of the molecule renders it incapable of inhibiting

trypsin and chymotrypsin, whereas other modifications affecting other amino acids leave its function intact, the lysyl bond is believed to be the reactive inhibitor site, at least for these two proteases (53). Cohen et al. (54) proposed that a Lys-Thr bond is cleaved during base catalyzed disruption of the complex and that a new carboxyl terminal lysyl residue is formed. These workers disrupted the complex using ^{18}OH labeled base. The new carboxyl terminal lysyl residue became labeled with ^{18}O. This conforms to the known distribution of oxygens which occurs during the base catalyzed hydrolysis of acyl esters, which would occur if the α_1-AT-trypsin complex were an acyl ester or if an acyl intermediate formed during the base catalyzed hydrolysis of a tetrahedral adduct. This led them to hypothesize that trypsin binds to a Lys-Thr bond near the amino terminal end of the α_1-AT. The bond is probably analogous to that usually formed between trypsin and its substrates. Since there are no intrachain disulfide bonds at the amino terminal end in α_1-AT, a tetrahedral complex is most likely. With an excess of elastase the α_1-AT molecule itself may be cleaved and inactivated; however, the peptide fragment remaining with the elastase molecole appears to inactivate it as well (55).

Physiologic Function of α_1-AT: The physiologic function of α_1-AT in the serum is unknown. It is not clear why there is an increase of this protease inhibitor in certain physiologic and pathologic conditions. The level of α_1-AT considerably increases in variety of conditions such as local inflammation, surgery, malignant tumors, injection of bacterial endotoxin, nephritis, pregnancy, and even during administration of

contraceptive hormones (56-60). The rise of α_1-AT concentration following trauma is quite steep and maximum values [twice the normal level] are reached in human subjects on the third day after gastrectomy (57) or after injection of typhoid vaccine (60). Heterozygotes for the deficiency gene have an elevation of α_1-AT but it reaches barely 50 percent of the response in normal individuals (56).

Recent work by Arora *et al.* (61) suggests that α_1-AT play an immuno-regulatory role in the suppression of antigen-dependent B-lymphocyte differentiation without affecting adherent or T-cells.

Pathogenesis of α_1-AT Deficiency: Severely deficient subjects of phenotype PiZ and probably PiSZ are much more susceptible to the development of emphysema or chronic bronchitis [or both] than the general population, the large majority of whom are of phenotype PiM (62,63). Patients of PiMZ phenotype are more likely to have symptomatic chronic obstructive lung disease than those of phenotype PiM (64). Larsson *et al.* (62) investigating aspects of lung function in smokers noted that abnormalities of elastic recoil, residual volume and closing capacity were significantly more prevalent in the PiMZ smokers than in the PiM smokers.

The experimental animal model to imitate natural emphysema closely was created by the intratracheal instillation of papain. Recently Fisk and Kuhn (65) observed emphysema-like changes in the lungs of the Blotchy mouse [Blotchy allele is one of several mutations at the Mottled locus on the mouse X-chromosome] and suggested Blotchy strain to be a useful model to investigate how abnormalities of connective tissue

proteins influence pulmonary structure and function. The discovery that emphysema often develops prematurely in persons who have virtually no α_1-globulin, a protein known to inhibit at least one proteolytic enzyme *in vitro*, directed investigators toward a search for a responsible *in vivo* enzyme. Proteases capable of producing emphysematous lesions in animal lungs were found in purulent sputum (66) and also extracted from the granules of polymorphonuclear leukocytes (23). Leukocyte elastase when instilled into the trachea of hamsters could produce the lesions of emphysema (67). Alveolar macrophages also released an elastase, but it was much less readily inhibited by α_1-AT than the elastase from leukocytes (68). Characteristically, the emphysema found in the lungs of PiZ cases is of panlobular type, although some patients do have the more common centrilobular variety (69).

In 1968, Sharp noted a serum electrophoretic pattern without an α_1-globulin peak and found it belonged to a child with cirrhosis (17). Microscopic examination of biopsy material showed that the globules in the hepatocytes readily combine with fluorescent-labeled antibody to human α_1-AT (70-72).

Those who have studied the frequency of α_1-AT deficiency in hepatic cancer have found that 10 to 50 percent of patients with hepato-cellular carcinoma have the characteristic globular bodies in their livers, although usually not in the tumor cells themselves (73-75).

Although proteolytic enzymes have access to several other tissues and could conceivably cause disease in them also if inhibition were ineffective, convincing data relating α_1-AT deficiency to any organ

other than the lung and the liver are sparse. Chronic pancreatitis or pancreatic fibrosis is only suggested by a few reports (76-78). Data in rheumatoid arthritis are conflicting (79,80).

Sporadic cases of glomerulonephritis in α_1-AT deficiency with liver disease have been reported (17), and the deposits containing α_1-AT found along the basement membrane have led to speculation that leakage of Z protein from dying liver cells may act as an antigen in the formation of immune complexes (81).

B. Trasylol

Historical Perspective: In 1925, Frey (82) discovered in mice a blood-pressure lowering substance of high molecular weight which was later termed Kallikrein from the greek name "kallikreas" for pancreas (83) and which today is included among what are known as the kininogenases. The first inhibitor for kallikrein was detected in the blood plasma by Frey and Kraut and was termed kallikrein inactivator (84,85). In 1930, the existence of a further kallikrein inhibitor in the bovine parotid, spleen, liver, and lymph nodes was verified by Kraut et al. (85). Astrup (87) found an inhibitor of fibrinolysis in extracts of the bovine lung.

Werle et al. (88) recognized that a connection exists between the inhibitors from the mentioned animal organs. He found that the kallikrein inhibitor he had described was also an inhibitor for trypsin and chymotrypsin, that the pancreatic trypsin inhibitor of Kunitz is also capable of inhibiting kallikrein (89), and that the inhibitor from the bovine lung has the same inhibitor specificity as the kallikrein-trypsin

inhibitor of the other bovine organs (90). Werle together with Marx was able to show that all these inhibitors also inhibit plasmin (90).

The inhibitor known as the kallikrein-trypsin inhibitor occurs only in some ruminants; in particular some bovine organs contain large quantities of it. It differs from the selective trypsin inhibitor which has been verified in the human, canine, porcine and bovine pancreas and which probably occurs in all mammals. It also differs from the inhibitor detected in the canine submandibuloris for trypsin and chymotrypsin and in the mammalian accessory genital glands for trypsin (91).
A significant distinguishing feature is that the inhibitory spectrum of the protease inhibitors of the serum (92) is different from that of the inhibitor from bovine organs (92). The inhibitors from the bovine parotid and lung are chemically identical (93). Many characteristics indicate that the kallikrein-trypsin inhibitors from other bovine organs, especially the liver, are no different from this inhibitor (92). The kallikrein-trypsin inhibitor from the bovine lung is industrially gained on a large scale in an almost pure form and is commercially available as Trasylol® [Bayer, Germany].

In 1936, Kunitz (86) isolated a basic trypsin inhibitor from bovine pancreas which--as we know now from the work of many different laboratories--is identical with Trasylol. The basic trypsin inhibitor Trasylol is not identical with the strongly monospecific trypsin inhibitor which occurs in the pancreas of all mammals. Besides the specific trypsin inhibitor bovine pancreas also contains the polyvalent inhibitor Trasylol. Furthermore, in contrast to the specific trypsin inhibitor,

Trasylol is secreted neither into the pancreatic juice nor saliva. It has been functionally recognized as an intracellular effector, the specific target of which is hitherto unknown (94).

Isolation: A process for isolating the kallikrein-trypsin inhibitor consists of alcohol-fractionation of the tissue extracts containing the inhibitor and subsequent paper-electrophoretic purification (95). From dealbuminized tissue extracts the inhibitor can be bound and fractionated to cation exchanges (96). Precipitation and selective adsorption methods, in conjunction with exchanges chromatography, are likewise applied for its isolation on a fairly large scale. The inhibitor can be brought to crystallization by salting it out (95). In this form, and also after the chromatographic purification, the action of 1 KIU [Kallikrein Inhibitor Unit] is bound to 0.14-0.15 μg of organic substance.

Biochemical Characterization: Biochemists have focused on the polypeptide Trasylol structure, its amino acid composition and sequence, its inhibitory spectrum, and on the mechanism of its inhibitory action. Common features of the enzymes inhibited by Trasylol are a serine residue in their active center. This is also true for kallikrein from pig pancreas, the active center of which, according to the studies of Fiedler et al. (97), is the sequence Asp-Ser-Gly.

The condition for the elucidation of the amino acid composition of a polypaptide like Trasylol is its isolation in sufficient amounts in a pure state.

Kunitz (86) prepared this inhibitor in a relatively pure form elegantly by binding it to pure trypsin and splitting the complex by trichloro-acetic acid. After further purification the amino acid composition and sequence of this preparation were examined by Laskowaski and Laskowaski (94,98). He found 58 amino acids and 4 amide groups. The molecular weight calculated from these results was 6513. The amino acid sequence and the position of the S-S bridges (Figure 1) were

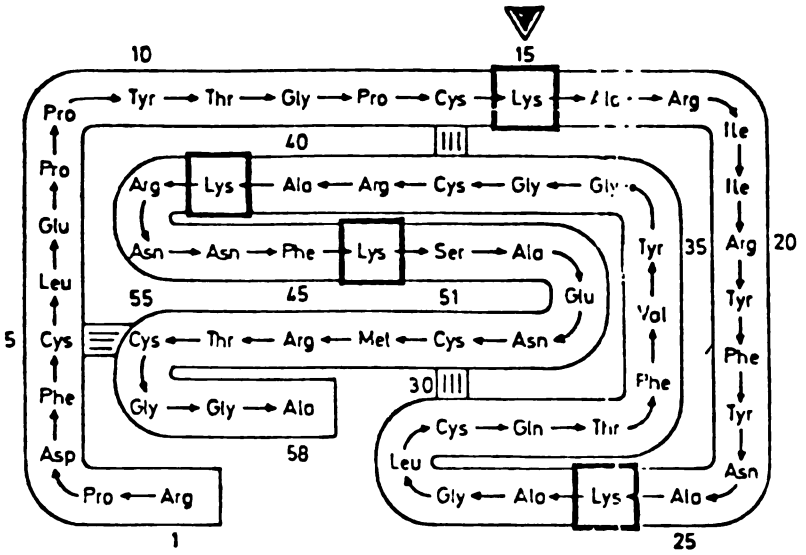

Figure 1. Amino acid sequence and S-S-bridges of the polyvalent prolease inhibitor (Trasylol) from bovine pancreas, respectively bovine lung. (Source: Figure 2 from Reference #112.)

clarified nearly at the same time by Anderer and Hornle (99) and by Laskowski (100). A special difficulty in these efforts was the fact that at that time no enzyme was known which could attack Trasylol. In the meantime we know from the work of Kassell (101) that thermolysine is able to split Trasylol at 60-80^{o}C into several peptides.

Protease Binding Site: The work of Archer (102) and of Laskowski Sr. and Jr. and their groups (100) revealed the importance of the free NH_2 group of the lysine 15 residue for the inhibitory activity against trypsin (98). Fritz and co-workers showed that this is true also for the inhibition of chymotrypsin and kallikrein (98).

Three-dimensional Structure: The crystallization of Trasylol by Schultz and Kraut (103) opened up the possibility of elucidation of the 3-dimensional configuration of Trasylol. This was performed by Huber et al. (194), after isomorphone crystallization with primarily aromatic mercury containing compounds.

Shape: According to Huber (104) the Trasylol molecule is pear-shaped (Figure 2).

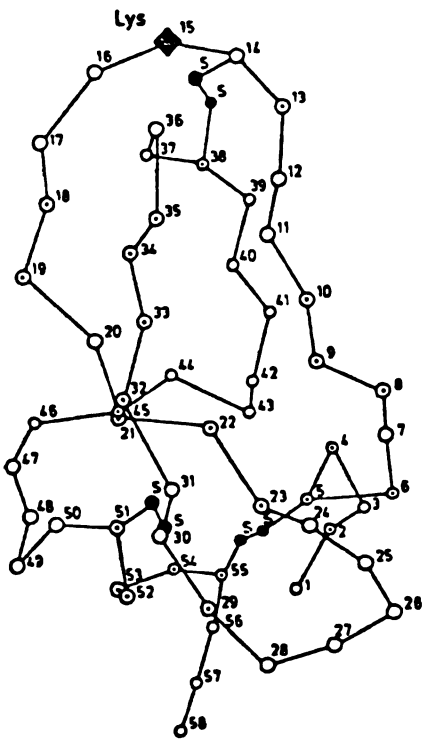

Figure 2. Three-dimensional structure of the polyvalent prolease inhibitor (Trasylol) from bovine lung. (Source: Figure 4 from Reference #112.)

Isoelectric Point and Stability: The isoelectric point is at pH 10.5 on account of its slight molecular size, the inhibitor is dialysable and not precipitable by de-albuminizing reagents.

The inhibitor is remarkably stable in the neutral and particularly in the acid range, even at fairly high temperatures; and contrary to what was formerly assumed it is also relatively insensitive in the alkaline range (105). The lysine 15 residue is situated at the top of the molecule in an extremely exposed position. Most of the basic positively charged amino acids are found in the upper half of the molecule whereas the acidic negatively charged ones are located at the base of the molecule.

Enzyme-Inhibitor Interaction: According to Huber (104) the exposed situation of the reactive center and the high dipole moment are of importance for the rapid and correct orientation of the inhibitor to the active center of mutual actions of enzymes and this inhibitor, salt bridges and hydrophobic effects are of special importance. They may explain the high affinity between inhibitor and enzymes. The lysine 15 side chain of Trasylol fits into the binding product, e.g., of trypsin (104) like a lysine containing substrate and in this way blocks the activity of the enzyme (Figure 3, on the following page).

The inhibition of trypsin and chymotrypsin by Trasylol is permanent. There is no splitting of a peptide bond which is the case with many other neutral inhibitors of trypsin. In an acidic medium the inhibitor can be regenerated from the complex without any alterations.

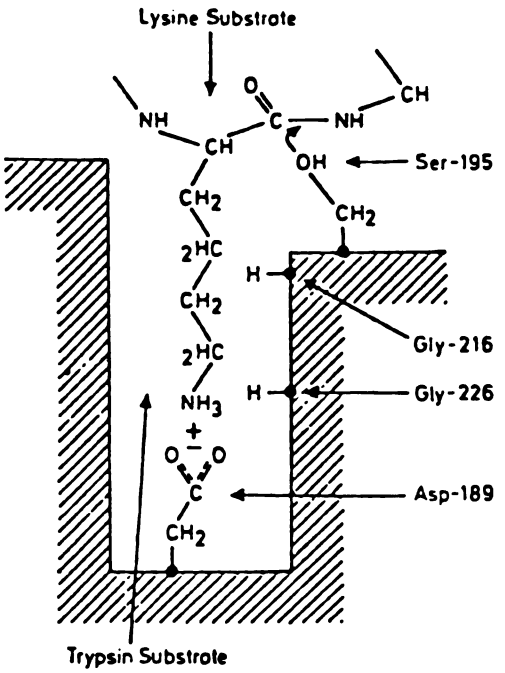

Figure 3. Binding pocket of trypsin with a lysine containing substrate for trypsin. (Source: Figure 5 from Reference #112.)

Inhibition Spectrum of Trasylol: The polyvalent inhibitor inhibits several enzymes of different substrate specificity, which however have in common the esterolytic and proteolytic activity and partly also the kinin-liberating activity. The inhibition extends to trypsin, chymotrypsin, the different kinds of kallikrein, plasmin as well as some bacterial proteinases.

Trypsin: The reaction of trypsin with Trasylol is pH-dependent above pH 11 and below pH 6, and it is reversible. In each case the proteolytic, esterolytic and kinin-liberating activity is equally strongly blocked. With trypsin the inhibition is linear with increasing inhibitor quantities only up to a limit value which lies between 70 and

90% inhibition. In the physiological pH range, 1 KIU inhibits the activity of 0.56 to 0.7 μg of trypsin. The dissociation constant of the trypsin-inhibitor complex is extremely low. For pH 7.8 an inhibitor constant is 2×10^{-11} mol/l. The dissociation of the complex increases as the pH decreases, i.e., the inhibitor constant becomes larger [at pH 4.0, e.g., the Ki = 2.6×10^{-9} mol/l] (105).

Chrmotrypsin: 1 KIU of Trasylol inhibits the activity of 0.44 μg of chymotrypsin. Expressed in unity of weight, the ratio of the trypsin to the chymotrypsin inhibition is 1:0.68. A dissociation of the chymotrypsin inhibitor complex occurs likewise in the acid range, the dissociation constant being, however, considerably greater than that for the trypsin-inhibitor complex. In a mixture of trypsin and chymotrypsin, trypsin is preferentially inhibited by Trasylol (105).

Kallikrein: The kallikrein-inhibiting capacity of the inhibitor is of special significance, because it was the starting point of the entire development in the field of Trasylol.

While the kallikrein-trypsin inhibitor is capable of inhibiting the kinin-liberating [proteolytic] and esterolytic activity of the kallikreins, the trypsin inhibitors from soya beans and ovomucoid inhibit only the serum kallikrein. Kallikrein-inhibitor complex has a relatively high dissociation constant [at pH 7.8 the Ki = 1.2×10^{-8} mol/l]. At pH 4.0 the kallikrein-inhibitor complex is already completely dissociated. Since the inhibitor is more firmly bound to trypsin than to kallikrein, when trypsin is added to the kallikrein-inhibitor complex, it displaces the kallikrein from its bond with the inhibitor. Once the

trypsin-inhibitor complex has formed, it is no longer segregated by the addition of kallikrein even when this kallikrein is added in large surplus quantities (105).

Plasmin: Measured in terms of splitting synthetic substrates, 1 KIU inhibits the activity of 6 μg of plasmin. Pronase-P is a proteinase mixture from Streptomyces griseus. 3.75 μg of the enzyme is inhibited by 1 KIU. Papain is inhibited by Trasylol only to a barely measurable extent [1 KIU inhibits 0.002 μg of papain]. Pepsin is neither inhibited by Trasylol nor is Trasylol attacked by pepsin (105).

Distribution and Execretion of the Inhibitor: After injecting the rat with 12000 KIU/kg, Trautschold and Werle (89) examined the distribution of the inhibitor over the individual organs. The inhibitor was initially found in slight quantities in all organs, then it selectively concentrated in the liver, with a maximum 30 min after the injection. After a latent period of about 10 min, the inhibitor content in the renal tissue began to rise; after 1 h it was 50% whereas only about 10% were still present in the blood. 4-5 h after the injection, almost the entire quantity of inhibitor administered was found in the kidneys.

Tests undertaken with Trasylol using tritium as tracer (90) showed that the inhibitor is excreted in a biologically effective form only to a slight extent in the course of several days; the main quantity appears only as tritium activity in the urine. From this, one may conclude that there is extensive decomposition of the inhibitor in the renal tissues.

Trasylol--as polypeptide with a relatively low molecular weight--is distinguished by a very good toleration even in high dosage.

The mouse LD_{50} is about 2.5×10^6 KIU/kg (106). The dog tolerates i.v. doses up to 1×10^6 KIU/kg without complications. Rats tolerate high Trasylol doses less well; especially operationally damaged animals may show severe shock-like reactions with high dosage (107). This is one of the reasons why the rat is ill-suited for tests with Trasylol. In rare cases, patients with a history of allergy can after repeated applications show intolerance reactions (106).

Pharmacology of Trasylol: Besides its capacity of inhibiting serine enzymes, Trasylol has another kind of activity: it may exert pharmacological actions. Pauschinger et al. (108) found that Trasylol accelerates the blood flow in the calf of the leg of man by arterial dilation and removes constriction. This observation was corroborated by Breght and co-workers (109) who studied in vitro the effect of Trasylol on the tonus of peripheral arteries and veins from cattle and of veins from the human leg. They found that in very low concentrations [10^{-6} to 10^{-8} g/ml] Trasylol relaxes the arteries and constricts the veins. The mechanisms of these effects is unknown.

The inhibiting effect of Trasylol in the development of edema in rat paws according to Kaller (110) could be elicited besides by inhibition of kinin liberation also by a direct permeability reducing action of Trasylol on the proximal capillaries in the inflamed area. Also the distribution of Trasylol in the body after i.v. injections shows some specificity of the affinity of Trasylol to special organs or membranes (111). They could show that Trasylol also stabilizes the membranes of lysosomes of leukocytes and inhibits their degranulation.

Trasylol as Therapeutic Agent: Trasylol was employed therapeutically for the first time in 1953 as a trypsin inhibitor on the suggestion of Frey and Werle (112, 113). Werle (112) reported large series of patients having acute pancreatitis who were treated with Trasylol. Thereafter, the favorable therapeutic action of Trasylol was confirmed in numerous clinical reports. The first attempt to use Trasylol therapeutically was based on the assumption of a premature activation of trypsin during acute pancreatitis. Though it was possible to demonstrate active trypsin in pancreatitis, it must be assumed that intermediary active trypsin acts as a primary activator of other pancreatic enzymes in cellular micro-areas.

There result four points at which Trasylol exerts a therapeutic action:

1. By inhibition of locally activated kininogenases Trasylol prevents the spreading of edema and stasis. This is caused by the action of kinins which increase vascular permeability, and dilate capillaries.
2. There occurs a partial inhibition of the reactions which lead to activation of the proteolytic system.
3. Under the influence of Trasylol a diminution of the secretory rate and secretion of pancreatic enzymes occurs.
4. Generalized liberation of kinins which leads to circulatory shock is prevented.

Trasylol should be administered in the very beginning of acute pancreatitis so that it may act on the initial edema.

Further indications for therapeutical use of Trasylol are chronic relapsing pancreatitis, postoperative pancreatitis, various forms of shock, e.g., during peritonitis, and large tissue damage. Activation of proteolytic systems in blood as it occurs in hyperfibrinolysis, and with activation of the thrombin system may also be influenced by Trasylol.

Trasylol and the Immune Response: Few studies have investigated the regulation of immune response by Trasylol. Prokopenko and Drobyazgo (114) studied the effect of Trasylol on antibody formation under normal conditions and in experimental atherosclerosis. They observed that Trasylol sharply inhibits the formation of specific immunoglobulins [especially 7S] in rabbits with experimental atherosclerosis but does not affect antibody formation in healthy animals. These authors proposed that some form of equilibrium arises in rabbits with experimental atherosclerosis between the immuno-depressant effects of cholestrol or its metabolis products and the immunostimulant action of a serum factor which is probably a proteolytic enzyme or its activator. Trasylol disturbs this equilibrium and thus causes inhibition of antibody biosynthesis in animals with experimental atherosclerosis.

While studying the interaction between lymphocytes and inflammatory exudate cells, Nakamura and his co-workers (115) found that the supernate of in vitro cultivated polymorphonuclear leukocytes [PMN-SUP] contain a factor which has an enhancing effect on the thymocyte-response. Thymocyte-helping potency of the SUP was abolished by adding Trasylol in soluble form or by passing SUP through a Trasylol affinity column. These authors thus assumed that the enhancing effect of PMN on

thymocyte-proliferative response was associated with the function of a neutral protease released from the cells, because the hemoglobinolytic activity of lymphocyte helping fraction was also inhibited when pre-incubated with Trasylol. They termed this protease, a lymphocyte helping protease [LHP].

More recently, Higuchi et al. (116) have described the inhibitory action of Trasylol on antigen or mitogen-induced lymphocyte triggering. By the use of incorporation of radiolabeled thymidine, uridine, and leucine into mouse lymphocytes, these workers observed that DNA synthesis, as well as RNA and protein syntheses are effectively inhibited by $0.3\text{-}2.5 \times 10^{-7}$ mol of Trasylol when responses are induced by homologous antigen, allogeneic cells, phytohemagglutinin [PHA] of Escherichia coli. DNA synthesis by splenic cells is not inhibited by Trasylol when the cells are stimulated with a relatively large amount of Concanavalin-A [Con-A]. Furthermore, the antigen-induced DNA synthesis by nonadherent lymph node cells is enhanced by the culture supernatent of macrophages, and this helping effect of macrophage supernatant is effectively inhibited either by soluble or insoluble Trasylol. Their observations lead them to the proposal that the inhibitory action of Trasylol on lymphocyte triggering operates indirectly interfering with the helping action of macrophages on lymphocytes.

II. Proteolytic Enzymes

The role of proteolytic enzymes in cellular functions has been expanded in recent years. Proteases have been found on the surface and

in the medium of cultured cells (4). There are also extensive reports that proteases produce a wide variety of effects on lymphocytes. Among these effects are: an alteration of electrophoretic mobility (5), a change in the pattern of homing, i.e., the migration of lymphocytes in vivo (6), an unmasking of certain alloantigens or an increase in antigenicity (117), and an alteration in the binding of antilymphocyte serum [ALS] (5). Recently, Hatcher et al. (118) have isolated a cytotoxic protease from human lymphocytes. The work of Hirschhorn et al. (10) suggests that an intracellular protease is involved in the blastogenic response of lymphocytes to PHA. The same authors also demonstrated that protease inhibitors like epsilon-amino caprioc acid and TLCK could suppress the lymphocyte response to PHA. Saito et al. (119) also reported that another protease inhibitor leupeptin suppress lymphocyte transformation and agreed with the previous authors that the inhibition of lymphocyte transformation was due to the inhibition of an intracellular protease.

Serine Proteases: Most of the proteases inactivated by protease inhibitors, come under the trypsin family.

This class of serine proteases is defined by the presence of a uniquely reactive serine side chain which makes a covalent ester bond to the carbonyl carbon atom of the susceptible bond in substrates to form an acyl-enzyme, and which reacts similarly with a number of covalent inhibitors (120). Two families of trypsin and subtilin have been well studied. Members of the trypsin family include: trypsin, chymotrypsin, elastase, plasmin, thrombin, collagenase and proteolytic enzymes of the complement system.

Mechanism of Protease Action: The mechanism of action of serine proteases on their specific substrates is generally formulated according to the following scheme:

$$E + I \rightleftharpoons [EI] \rightleftharpoons [EI]^{*} \rightleftharpoons [EI]^{**} \rightleftharpoons [EI']^{*} \rightleftharpoons E + I'$$

Present evidence (121,122) suggests that protease inhibitors function in a role analogous to that of a substrate. $[EI]^{*}$ represents an inactive complex in the form of a tetrahedral adduct which in the case of STI and trypsin involved arginine-63 of STI and active site serine residue of trypsin (123,124). This complex can be dissociated, however, by kinetic control (125) to yield primarily the virgin inhibitor I. The existence of this postulated acyl-enzyme intermediate was resolved by Huang and Liener (126). They presented the evidence indicating that a small but detectable fraction of the STI-trypsin complex does in fact exist in the form of acyl intermediate [ester bond involving the carboxyl group of arginine-63 and the active serine residue of trypsin] in equilibrium with the predominent tetrahedral species. This demonstration was facilitated through the use of ^{125}I-labeled STI and the subsequent "trapping" of the acyl-enzyme intermediate.

Bode et al. (127) using X-ray crystallography compared the structures of the free components with the molecules in the complex and observed the structures of stable intermediates of proteolysis. Their studies indicated that complex forms a tetrahedral adduct with a long bond of 2.6^{o}A between C of lys 15 [inhibitor] and O^{γ} of serine 195.

C of lys 15 [inhibitor] is strongly tetrahedrally deformed with the carbonyl oxygen -34^{o} out of the plane formed by N, C, $C^{\alpha 3}$.

This agrees with NMR-studies on the complex, and suggests that a considerable degree of proton transfer from Ser 195 to His 57 occurs in the complex (127).

In view of the functional similarities of all protein protease inhibitors, Bode et al. (127) believed that their association with trypsin follows the same stereo-chemical principles. These workers also noted that complexes with other members of the trypsin protease family resemble each other closely due to the strongly conserved active site and substrate contact segments.

Kaplan and Bona (128) have shown the mitogenic effects of proteases such as pronase and trypsin. It can be postulated that mitogens and antigens cause the release and activation of an endogenous protease at the lymphocyte surface whose proteolytic activity is essential for the subsequent events of blast transformation.

Neutral proteases such as trypsin, chymotrypsin, pronase and pancreatic elastase have been shown to induce lymphocyte transformation and proliferation (7). Grayzel et al. (129) even have found a neutral protease bound to lymphocyte cell membrane.

The extent to which proteases exist bound to the surface of cells or secreted by them remains to be determined. The function of a surface protease is a matter of conjecture but its presence on the lymphocyte, a cell whose surface is the site of considerable cellular activity including the migration and shedding of receptors and the secretion of

immunoglobulins, suggests that proteases may play a role in the dynamic processes of the lymphocyte surface membrane. It is quite conceivable that cell surface or exogenous proteases may also be mitogenic and thus have a role in the initial differentiation of lymphoid cells, since they short-circuit the endogenous proteolytic step supposed to be common to all blast transformation.

Observations that both exogenous and endogenous proteases have a role in the lymphocyte blast transformation, and also the studies that protease inhibitors like TLCK and leupeptin can suppress the action of such proteases, lead to the hypothesis that protease inhibitor present in the normal serum, may have its immunoregulatory effect on lymphocytes in two ways:

1. Protease inhibitor blocks the mitogenic effect of cell surface proteases and/or exogenous proteases thereby preventing the initial differentiation of lymphocytes [i.e., before exposure to the antigen].
2. It affects the proteolytic activity of endogenous proteases released on the lymphocyte surface after exposure to the antigen. By this action, protease inhibitor prevents the subsequent events of blast transformation.

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ARTICLE 1

$ALPHA_1$-ANTITRYPSIN IS AN EFFECTOR OF IMMUNOLOGIC STASIS

ALPHA$_1$-ANTITRYPSIN IS AN EFFECTOR OF IMMUNOLOGIC STASIS

A number of proteins present in normal serum can serve as suppressors or enhancers of the immune response. Recently an α-globulin rich fraction of Cohn Fraction IV, designated IRA (immune-regulatory α-globulin), has been implicated in suppressing the *in vitro* antibody response of mouse spleen cells to sheep red cells without cytotoxicity.[1] Studies by Chase[2] also indicate that α_2-macroglobulin, one of the protease inhibitors present in normal serum, limits the human lymphocyte response to phytohemagglutinin, and Concanavalin-A as measured by ^{3}H-thymidine incorporation. A similar role for α_1-antitrypsin (α_1-AT), the major protease inhibitor in normal serum, can be postulated to be involved in the regulation of the immune response. Proteases have been found on both the surface and in the medium of cultured cells[3]; they have also been implicated in altering the electrophoretic mobility,[4] *in vivo* migration[5] and blast transformation[6] of lymphocytes.

The physiologic function of α_1-AT in the serum is unknown. It is not clear why there is an increase of this protease inhibitor in certain physiologic and pathologic conditions.[7] Results of the present communication suggest that α_1-AT plays an immunoregulatory function by having a suppressive effect on the *in vivo* and *in vitro* immune response of mouse spleen cells against sheep red cells (SRBC). For these studies spleens from 9-16 week old hybrid C57BL/10XC3H ($BC3F_1$) female mice were dissected free of the surrounding fascia and cells were expressed from

the capsules. Single cell suspensions were gently aspirated and expelled by syringe through needles of 21 and 27 gauge. The cells were washed once then resuspended in spleen cell medium.[8] Viability was checked by the Trypan blue exclusion method.

α_1-AT isolated from normal human plasma was obtained from Dr. Y. L. Hao (American National Red Cross, Blood Research Center, Bethesda, Maryland 20014) and from Dr. Charles B. Glazer (The Institute of Medical Sciences, San Francisco, California 94115). α_1-AT activity was determined by measurement of its trypsin inhibitory capacity[9] and quantitatively confirmed by radial immunodiffusion.[10] It was also found to have a normal M1 phenotype pattern by isoelectric focusing[11] and by disc gel electrophoresis.[12]

Immunoregulatory effects of protease inhibitors were studied *in vivo* by injecting various concentrations of α_1-AT into groups of BCF_1 mice. α_1-AT ranging from 250 µg to 1000 µg was given intravenously into mice along with 10^7 SRBC. The control groups received either human serum albumin (HSA), or human gamma globulin (HGG). Results presented in Table 1 indicate that these concentrations of α_1-AT significantly suppressed the plaque response. Furthermore, as the dose of α_1-AT was increased, greater suppression of PFC response resulted.

Next α_1-AT effects on the immune response were studies *in vitro* by adding various amounts of this protease inhibitor to spleen cell cultures. 2×10^7 spleen cells ml^{-1} with 0.05 ml of 1.5 percent SRBC were cultured in Marbrook vessels at $37^{\circ}C$ for 5 days at 8% CO_2. The control group received either HSA, HGG, or an equivalent amount of medium. Hemolytic

plaque assays for anti-SRBC producing cells (PFC) were conducted using a modified slide method of Mishell and Dutton.[13] At a concentration of 100 µg/culture, α_1-AT significantly reduced the plaque response to 49% of the control. Increasing concentrations of α_1-AT from 100 µg/culture to 1000 µg/culture resulted in even greater suppression of PFC responses (data not presented). α_1-AT had no effect on plaque response in concentrations ranging from 1-50 µg/culture.

The following are summarized control results (data not presented here). HSA, HGG or acid glycoprotein fraction (prepared according to the method of Hao et al.) served as controls and had no effect upon spleen cell culture responses to SRBC. Also, α_1-AT was not demonstrated to bind to antigen, and it did not lose biologic activity under described culture conditions. To exclude the possibility that α_1-AT suppression was due to an effect on the antigen, SRBC were pretreated with α_1-AT, washed and then added to the cultures. Results indicated that suppression by α_1-AT was not due to an effect on the antigen.

Kinetic studies (Table 2) indicate that 48 hours exposure to α_1-AT was sufficient time to produce a reduction in plaque number which decreased even further when spleen cells are exposed to α_1-AT for up to 5 days. To investigate whether immuno-suppression by α_1-AT was a result of interaction with the adherent cell population (phagocytic cells) of spleen, groups of cultures were incubated with either α_1-AT or an equivalent amount of culture medium. After 72 hours, non-adherent cells were separated from adherent cells by repeated washing of the monolayers.

Combinations of treated and untreated adherent and non-adherent cell populations were arranged as shown in Table 3. Results of anti-SRBC

responses by these cultures indicate that immuno-regulation by α_1-AT was not due to an effect on the adherent cells. To further determine the lymphoid cell type regulated by α_1-AT, groups of spleen cell cultures were set up as before. After separating the non-adherent cells by repeated washings of the monolayer, the non-adherent cells were further separated into T-cells and B-cells, by passage over nylon-wool columns. The combination of T-cells, B-cells and adherent cells was made as described in Table 4. These studies indicate that α_1-AT does not affect adherent cells or T-cells. It does, however, result in a regulation of antigen dependent B-cells responses.

In conclusion, with no outward effect on cell viability, α_1-AT significantly lowers the immune response both in vitro and in vivo as measured by the number of plaques produced by spleen cells against SRBC. Immunosuppression by α_1-AT is not due to an effect on the antigen. Furthermore, in these studies, α_1-AT did suppress antigen dependent B-cell differentiation without affecting adherent or T-cells.

Work is presently in progress to further determine the mechanism of action of α_1-AT. Of particular interest is its regulatory effect on cellular proteases. The information obtained through such studies will contribute to our understanding of the molecular event in lymphocyte maturation. In addition to providing basic insight to early events of cell differentiation, these studies may further elucidate the association between proteinase inhibitor and immune deviation and the relationship to various disease states.

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TABLE 1

EFFECT OF α_1-AT ON THE ANTI-SRBC RESPONSES OF MICE

Treatment*	Dose µg/mouse	Anti-SRBC Responses** (PFC/spleen x 10^3)+	
		Direct	Indirect
α_1-AT	1000	75 ± 4	2 ± 0.28
α_1-AT	500	85 ± 11	3 ± 0.8
α_1-AT	250	96 ± 5.2	9 ± 2.6
HSA	1000	139 ± 7.4	17 ± 2.4
---	---	156 ± 16	19 ± 5.5

* Each mouse also received 10^7 SRBC by intravenous route.

**A 0.05 percent solution of agarose in minimal essential medium with Hanks base was distributed in 0.4 ml aliquots into prewarmed Wassermann tubes in 53°C. 0.05 ml of one-fourth (v/v) SRBC suspension and spleen cells (0.1 ml) were added and the mixture then spread on a slide precoated with 0.1 percent agarose. After one hour of incubation at 37°C in humid 8% CO_2 atmosphere, the slides were treated with 1:10dilution of guinea pig serum as a source of complement. The slides were incubated for another 2-3 hours and PFC counted.

+ Mean ± standard error of 4 mice.

TABLE 2

KINETICS OF α_1-AT ON IN VITRO ANTI-SRBC RESPONSES*

Addition	Exposure Time of Culture to α_1-AT (# days)	Anti-SRBC Responses (PFC/culture)**
α_1-AT	5	320 ± 43
α_1-AT	4	395 ± 57
α_1-AT	3	633 ± 41
α_1-AT	2	841 ± 71
α_1-AT	1	1251 ± 120
---	---	1229 ± 96

* Spleen cell cultures of 2×10^7 cells with SRBC

** Mean ± standard error of 5 cultures

TABLE 3

α_1-AT EFFECT ON ADHERENT AND NON-ADHERENT SPLEEN CELLS FOLLOWING ANTIGEN STIMULATION

Groups	Anti-SRBC Responses (PFC/culture)**
A* + NA	564 ± 36
A + NA*	161 ± 11
A* + NA*	33 ± 5
A + NA	599 ± 50

* α_1-AT treatment

** Mean ± standard error of 5 cultures

TABLE 4

THE IMMUNOBIOLOGICAL EFFECTS OF α_1-AT ON T-CELLS, B-CELLS AND ADHERENT CELLS OF SPLEEN

Groups	Anti-SRBC Responses (PFC/culture)**
T* + B + A	515 ± 13
T + B* + A	238 ± 19
T* + B + A*	380 ± 28
T + B* + A*	165 ± 9
T* + B* + A*	69 ± 18
T + B + A	552 ± 51

* α_1-AT treatment

** Mean ± standard error of 5 cultures. Cultures in the various groups consisted of 10^7 T-cells and 10^7 B-cells.

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ARTICLE 2

PROTEASE INHIBITOR REGULATION OF B CELL DIFFERENTIATION[1]

I. The Effect of Trasylol on the Primary and Secondary Antibody Response

Running head: Trasylol Suppression of B Cells

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FOOTNOTES

1. This work was supported by grants from the National Institutes of Health (CA-13396 and AI-12549), and the American Cancer Society (IM-158).

2. H. C. M. is the recipient of an American Cancer Society Faculty Research Award (FRA-147).

3. Abbreviations used in this paper: PFC, plaque-forming cells; P.I., protease inhibitor; α_1-AT, α_1-Anti-trypsin; SRBC, sheep erythrocytes; FCS, fetal calf serum; MEM, minimal essential medium; PBS, phosphate-buffered saline; HSA, human serum albumin; KIU, kallikrein inactivating unit.

INTRODUCTION

Protease inhibitors (P.I.) are known to have a function to inhibit proteolytic enzymes (e.g., elastase; cathepsin-G) produced by leukocyte granules (1). Furthermore, they also inhibit proteases produced by certain bacteria during their infection (2). More recently N-α-tosyl-L-lysyl chloromethyl ketone (TLCK), a protease inhibitor, has been shown to affect an intracellular protease thought to be responsible for lymphocyte blastogenesis (3). A similar role for Trasylol, a polyvalent enzyme inhibitor isolated from bovine lung, can be postulated to be involved in the regulation of the immune response.

Trasylol (aprotinin) originally isolated by Frey (4), Kraut et al. (5), and Kunitz and Northrop (6), has been widely used in the treatment of hemorrhages caused by hyperfibrinolysis and pancreatitis, where it inhibits excessive activation of certain proteinases which occur during the development of the disease. Pharmacologically, this kallikrein inactivator has been shown to dilate arteries and constrict veins (7), prevent edematous development in the inflamed area of rat paws (7) and when injected intravenously, shows binding for special organs or membranes (8,9).

Prokopenko and Drobyazgo (10) observed that Trasylol could inhibit the formation of 7S immunoglobulins in rabbits with experimental atherosclerosis. They proposed that Trasylol was inhibitory to the

immunostimulant factor that circulated in the blood of experimental atherosclerotic animals.

Nakamura and co-workers (11) observed that Trasylol could abolish both the proteolytic activity and thymocyte helping potency of SIP [supernatent of mouse polymorphonuclear (PMN) leukocytes cultivated *in vitro*].

The studies of Higuchi *et al.* (12) indicate that Trasylol, reversibly inhibits DNA, RNA as well as protein synthesis of lymphocytes triggered by homologous antigen, allogeneic cells or mitogens. These workers proposed that the Trasylol inhibition of lymphocyte triggering could be due to its interference with the helper action of macrophages on lymphocytes.

The role of proteolytic enzymes in cellular functions has been expanded in recent years. Proteases have been found on the surface and in the medium of cultured cells (13,14,15) and inhibitors of proteolytic enzymes have been shown to affect such activities of cells as growth (16,17) and the response to plant lectins (3).

While much is now known about its chemical nature (a small peptide, MW 6512), *in vivo* synthesis and potential therapeutic importance, the functional significance of Trasylol and the mechanisms involved in its regulation of the immune response is not understood. Since one of the major unique properties of Trasylol is its protease-inhibiting capacity, similar to that of α_1-antitrypsin (α_1-AT) one intriguing possibility is that Trasylol also has immunoregulatory properties.

During the course of our previous study (18) we proposed that α_1-antitrypsin, a constituent of normal human serum, has an

immunoregulatory junction based on demonstration of a non-cytotoxic immunosuppressive effect on the primary antibody response both in vitro and in vivo by this protease inhibitor. Furthermore, we observed α_1-AT suppression of antigen-dependent B-cell differentiation without affect on T-cells or adherent cells.

The present study extend this concept by examining the effect of a polyvalent enzyme inhibitor Trasylol on the primary and secondary immune response to both T-dependent and T-independent antigens. Further experiments have been done to investigate if immunoregulation by Trasylol is due to its protease inhibiting capacity, and finally to determine its mode of action.

MATERIALS AND METHODS

Animals: C57BL/10 x C3H/He (BCF_1) female mice were from Cumberland View Farms, Clinton, Tennessee.

CELLS FOR CULTURE AND ASSAY: Spleen cells were obtained aseptically from 9 to 16-week-old C3H x C57BL F_1 female mice (Cumberland View Farms, Tenn.). Cell suspensions were prepared by gentle aspiration with a syringe and needles of progressively increasing gauge (21 to 27) to obtain a single cell suspension. Spleen cells were washed once and resuspended in medium CMRL 1066 (Grand Island Biological Co., Grand Island, NY) supplemented with 15% fetal calf serum (FCS) (Grand Island Biological Co.), 0.15 mM L-asparagine, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 mg/l gentamycin. Spleen cells were cultured in 35 mm tissue culture dishes (Falcon Plastics, Division of BioQuest, Oxnard, CA).

Viability of cells was determined by trypan blue exclusion in all experiments.

TRASYLOL: Trypsin-Kallikrein Inhibitor (aprotinin) (FBA Pharmaceuticals, New York, NY) was diluted in spleen cell medium (pH 7.2-7.4) to obtain a desired concentration/culture.

TRYPSIN: 2x crystallized, dialyzed and lyophilized (Sigma Chemical Co., St. Louis, MO 63178) was also dissolved in spleen cell medium to obtain desired concentration.

ANTIGENS: Sheep erythrocytes (SRBC) were obtained from a single animal (Lot #8227121, Grand Island Biological Co., Grand Island, NY) and were stored in Alsever solution. Before use, the SRBC were washed three times in sterile phosphate buffered saline (PBS) and suspended to 1×10^9 cells/ml in spleen cell culture medium.

Clinical grade dextran with an average molecular weight of 2×10^6 (Sigma Chemical Co., St. Louis, MO 63178) was dissolved in pyrogen-free PBS at 20 mg/ml, filtered (Millipore 0.22 mM), stored at $-20^{\circ}C$, and thereafter thawed for dilution in spleen cell medium before use. Dextran was added to the cultures in concentrations and at times shown in tables. Dextran, in a wide range of doses, was neither mitogenic nor cytotoxic as evaluated by viable cell counts.

COUPLING OF DEXTRAN TO SRBC: For dextran coupling SRBC were washed three times in PBS, pH 7.4, and resuspended to a 50% cell suspension in the phosphate buffered saline. Two milliliters of dextran solution containing 10 mg/ml were added to 2 ml of 50% SRBC as reported by Ghanta et al. (19). After incubation with stirring at $37^{\circ}C$ the dextran-coupled SRBC (Dex-SRBC) were washed three times with PBS to remove any

unreacted dextran. The cells were resuspended in MEM to a final concentration of 1 x 10^9/ml, and stored at 4°C. Dextran-SRBC were prepared fresh a few hours before their use.

THYMECTOMY: Four-week-old mice were thymectomized, according to the methods of Miller (20). They were allowed to rest for at least one month prior to irradiation. When thymectomized mice were sacrificed, the mediastinum was examined microscopically for the presence of thymus remnants. No such remnants were found.

IRRADIATION: Ten- to 12-week-old mice received 900 Rads of whole body irradiation from the ^{60}COγ-irradiation source in the Department of Food Science at Michigan State University. The animals were rested for at least 4 hr before transplantation.

Cell Suspensions: Bone marrow cells from tibias and femurs of 10- to 12-week-old normal BCF_1 mice were gently aspirated in Eagles minimum essential medium (MEM) in Hanks' salts with a syringe and a 25 gauge needle. Cells were then passed through a 27 gauge needle to produce a dispersed cell suspension. The cell suspension was washed once by centrifugation (170 g) in MEM and was then diluted to the appropriate concentration to be injected. Each mouse received 1 x 10^6 bone marrow cells.

Steps for Separation of Cells

Macrophages: Spleen cells of normal mice were placed in tissue culture petri dishes (Falcon Plastics, Division of BioQuest, Oxnard, CA). After 24, 48 or 72 hours of incubation at 37°C, the non-adherent cells were separated from the adherent cells by repeated washing with fresh

spleen cell medium. The adherent cells were used as a source of macrophages.

T-Cells: One column was used for every 2 x 10^8 non-adherent cells. Before use, the conditioned (soaked in MEM + FCS, 37°C, 1 hr) nylon wool column was washed with 20 ml MEM + FCS. Resuspended non-adherent cells were poured over the nylon wool column, and then incubated for 45 min at 37°C. T-cells from the column were recovered by dropwise adding of 25 ml MEM + FCS.

B-Cells: After recovery of T-cells, the above column was washed with 50 ml of MEM + FCS and then pressed and squeezed to recover B-cells. This process of washing and squeezing the nylon-wool column was repeated a number of times to recover most of the B-cells. The B-cell preparation was further purified by treatment with mouse anti-theta serum and complement.

In order to determine which cell type is regulated by Trasylol, individual cell populations were pre-incubated with known amounts of Trasylol. An assay system containing no Trasylol was reconstituted with each cell population pre-exposed to Trasylol. Any change in the plaque response in comparison to the control group was recorded.

HOMOLYTIC PLAQUE FORMING CELL (PFC) ASSAY:

a. Primary In Vitro Antibody Synthesis:

Primary antibody synthesis to sheep erythrocytes (SRBC) was measured using the method described by Mishell-Dutton (21). Spleen cell suspensions of 2 x 10^7 viable cells (viabilities greater than 85%) in one ml of spleen cell culture medium and 0.05 ml of SRBC (1.5%) were

cultured for 4 days in 35 mm tissue culture dishes (Falcon Plastics, Division of BioQuest, Oxnard, CA) with rocking. Cultures were set up in a total volume of 1.15 ml in the following order: a. 0.05 ml of antigen, b. 2×10^7 spleen cells in 1.0 ml of medium, and c. Trasylol to be tested for immunosuppressive activity was added in 0.1 ml at the beginning of culture unless otherwise stated. After the spleen cell cultures were incubated for 4 days at $37^{\circ}C$ in a humid 8% CO_2 atmosphere, the cells from the tissue culture dishes were removed by gentle aspiration with a Pasteur pipette. At the time of assay, viabilities and cell concentrations in each experimental group were measured. There were an average of $2\text{-}3 \times 10^6$ viable spleen cells remaining in each group after 4 days of cultivation. No discernible difference in viability or concentration was detected when spleen cells were cultured with or without Trasylol. Suspensions from these spleen cell cultures (0.1 ml) were assayed by the Jerne hemolytic plaque method as modified for use with agarose gel on glass microscopic slides. Details of this procedure are described elsewhere (22).

The localized hemolysis-in-gel technique used was to detect and enumerate cellular synthesis of γM antibody [direct plaque forming cells (PFC)]. The antibody responses are expressed as mean PFC per culture $\pm$ SE (standard error of the mean). Each preparation was cultured as five cultures per group.

b. Secondary In Vitro Antibody Synthesis:

Secondary synthesis to SRBC was measured also by an adaptation of the Mishell-Dutton culture system (21). Groups of mice were injected

with 10^7 SRBC/mouse through their tail vein on day zero. After 14, 21 and 35 days, the primed spleen cells were cultured with and without the antigen as described in the previous section. Trasylol to be tested for immuno-suppressive activity was added in 0.1 ml at the beginning of culture unless otherwise stated. After the 4 day culture period in an atmosphere of 8% CO_2, at 37°C, the cells were removed from the tissue culture dishes by gentle aspiration with a Pasteur pipette. At the time of assay, viabilities and cell concentrations in each experimental group were measured. No discernible difference in viability or concentration was detected when spleen cells were cultured with or without Trasylol. Suspensions from these spleen cell cultures (0.1 ml) were assayed using the method described in the previous section. The antibody responses are expressed as mean PFC per culture $\pm$ S.E. Each preparation was cultured as five cultures per group.

RESULTS

Suppression of the In Vitro Antibody Response by Trasylol: In order to study the effect of Trasylol on the immune response, mouse spleen cells were cultured in tissue culture dishes with and without the antigen. Trasylol was added to spleen cell cultures at several concentrations. The plaque response of spleen cells against SRBC was examined after 4 days of incubation. Data shown in Table 1 indicates that when Trasylol was added at a concentration of 100 KIU/culture, the plaque response was reduced to nearly 50% compared with controls consisting of cultures to which normal human serum albumin (HSA) was added in similar

concentrations. The dose-response to Trasylol shown in Table 1 demonstrates that increasing the concentration of Trasylol from 250 to 2,000 KIU/culture reduced the PFC number to a significant degree when compared with the control group.

Effect of Pre-incubation of Spleen Cells with Trasylol on the Primary Antibody Response: 2×10^7 spleen cells in a volume of 1 ml were pre-incubated with 0.1 ml of 500 and 1,000 KIU of Trasylol for 3 and 6 hr. Cultures were then given repeated washings before exposure to the antigen. The primary PFC response was measured 4 days after addition of antigen. There was no measurable change in viability of control and treated cultures and no suppression evident in any of the cultures (data not shown) indicating that pre-incubation of spleen cells with Trasylol in cultures does not result in suppression of the primary PFC response.

Effect of Excess Antigen on Suppression of Primary Antibody Response by Trasylol: To determine if suppression of PFC response by Trasylol is due to some indirect effect on the antigen, the concentration of the antigen was increased from 1.5% to nearly 2.5% of SRBC. As noted in Figure 1, increase in concentration of the antigen (from 1.5 to 2.5%) in the culture system did not have any effect on the suppressive ability of Trasylol (63-180 PFC/culture), thus indicating that immuno-suppression by Trasylol observed (69 $\pm$ 14 PFC/culture) in the system is not due to its effect on the antigen.

Dose-response Effect of Trasylol on Memory: Experiments were done to determine whether Trasylol would exert suppressive effects on the

secondary antibody response. For this purpose, groups of mice were primed with 10^7 SRBC/mouse on day zero. After 14, 21 and 35 days of priming spleen cells were collected and cultured with SRBC and in the presence or absence of Trasylol (final concentrations ranging from 500 to 2,000 KIU/culture). As seen in Table 2 when SRBC-primed spleen cells were exposed to the same sensitizing antigen, there was a high response of plaque forming cells (>8,000 PFC/culture). Addition of Trasylol (500 KIU/culture) decreased the secondary plaque response to about 6,000 PFC/culture. Suppression of PFC response was even more profound if concentration of Trasylol/culture was raised to either 1,000 or 2,000 KIU. Thus, Trasylol significantly reduced ($P < .05$) the secondary antibody response in a dose-dependent manner.

Kinetics of the Effect of Trasylol on Spleen Cells Following Exposure to Antigen: It next became important to examine the suppressive effect of Trasylol at early as well as later stages of the immune response. The culture system (i.e., 2×10^7 spleen cells + SRBC) was incubated with Trasylol in a final concentration of 1,000 KIU/culture and Trasylol was removed at various intervals ranging from 6 to 96 hr later. The results of one of these time-kinetic experiments are presented in Figure 2. The data indicate a sharp decrease in PFC response between 18 and 24 hr after antigenic stimulation. The number of PFC/culture up until 18 hr of exposure to Trasylol was approximately 500 comparable to that of the control group which had 600 PFC/culture. However, at 24 hr there was a sharp decline (112 PFC/culture) which was significant at $P < .05$. Suppression of plaque number was more pronounced

($P < .01$) when spleen cell cultures were exposed to Trasylol for 2 to 4 days. In a similar fashion, Trasylol (1,000 KIU/culture) was added at various intervals ranging from zero to 96 hr after initiation of spleen cell cultures. Results (data not presented) indicated a pronounced suppression in the PFC response, if Trasylol was added any time before 72 hr of initiation of cultures. However, no suppression of PFC response occurred when Trasylol was added between 72 and 96 hr.

Reversal of Immuno-suppression upon Prolonged Incubation of Trasylol-treated Spleen Cell Cultures: The Trasylol effect could be reversed when this protease inhibitor was removed from spleen cells incubated with SRBC and in the presence of Trasylol (500 and 1,000 KIU/culture). After 24 hr of incubation, Trasylol was washed from the culture system and PFC response of pre-treated cultures was determined on 5, 6 and 7 days (prolonged incubation periods than usual). We observed (data not presented) that once Trasylol was removed from the system, and the cultures were left for prolonged incubation periods, immuno-suppression by Trasylol was reversed.

Effect of Trasylol on the Primary Anti-Dextran Antibody Response In Vitro: This protease inhibitor could also be shown to affect responses to other antigens. When the T-independent antigen Dextran (MW. 2×10^6 daltons) was tested, Trasylol, at a concentration below 100 KIU/culture, did not produce any change in the primary anti-dextran PFC response. However, at a concentration of 250 KIU/culture Trasylol suppressed the PFC response to nearly 50% of the control cultures. With 500 KIU of Trasylol/culture, there were approximately 650 anti-dextran

culture, compared to the control group with 1,606 ± 121 PFC/culture. This suppression of primary anti-dextran PFC response was even greater (410 ± 48 PFC/culture) when higher concentrations of Trasylol (1,000 KIU) were used per culture (Table 3).

We determined that the effect of Trasylol was directed primarily to those cells synthesizing anti-dextran antibodies by exposing spleen cells (from thymectomized, X-irradiated and bone marrow-reconstituted mice) to various concentrations of Trasylol in vitro. As presented in Table 3, an enriched B-cell population devoid of Thy-1 antigen bearing cells, responded less well (824 ± 47 PFC/culture) to Dextran, than did whole spleen cells (1,606 ± 121 PFC/culture). Once again, Trasylol, at a concentration below 100 KIU/culture, did not have any suppressive effect on the primary anti-dextran PFC response. On the other hand, with a concentration of 250 KIU or more, Trasylol suppressed the PFC response of spleen cells to Dextran.

Kinetics of the Trypsin Neutralization of Immunosuppression by Trasylol: The neutralizing effect of trypsin on the ability of Trasylol to suppress PFC response of spleen cells against SRBC is shown in Table 4. 500, 1,000 and 2,000 KIU of Trasylol were pre-incubated with different concentrations of trypsin (shown in Table 4) for 3 hr. After this time, Trasylol-trypsin mixture was added to spleen cell cultures. REsults presented in Table 4 indicate that immuno-suppression of PFC response by 500 and 1,000 KIU of Trasylol was abrogated partially by 50 and 100 µg of trypsin (1,153 ± 88 and 1,250 ± 65 PFC/culture respectively) and completely by 150 µg of trypsin (1,835 ± 78 PFC/culture).

Even 150 μg of trypsin did not abrogate the effect of 2,000 KIU of Trasylol. Control cultures with varying concentration of trypsin, exhibited plaque numbers ranging from 1,740 ± 138 to 2,114 ± 124 PFC/culture.

Effect of Trasylol on Select Cell Populations: It was next important to determine the cell population influenced by the protease inhibitor. Adherent or non-adherent cells were studied by incubation of groups of cultures with either Trasylol (1,000 LIU/culture) or an equivalent amount of culture medium. After 24, 48 or 72 hr of incubation, the non-adherent cells were separated from the adherent cells by repeated washing of the monolayers. Combinations of Trasylol-treated and untreated adherent and non-adherent cell populations were arranged as shown in Table 5. Results (Table 5) indicate that when adherent cells were exposed to Trasylol for 24 to 72 hr, there was no suppression of the PFC response (approximately 700 PFC/culture) when compared with the control group which went through the similar technical steps of cell separation. When only the non-adherent cells in the culture system were exposed to Trasylol for 24, 48 or 72 hr, the PFC response was suppressed to a significant degree ($P < .01$) when compared to the control group (684 ± 38 PFC/culture). Comparable suppression of plaque numbers was observed (124 ± 19 PFC/culture) when both cell populations were exposed to Trasylol and went through the same technical steps of cell separation.

Effect of Trasylol on T-cells, B-cells and Adherent Cells of Spleen Following Antigen Stimulation: Further determination of the lymphoid cell type affected by Trasylol were studied with T and B cell

populations. Groups of spleen cell cultures were sorted as adherent and non-adherent cells as before. The non-adherent cells were further separated into T-cells and B-cells by passage over nylon-wool columns. The combinations of T-cells, B-cells and adherent cells (A-cells) was made as described in Table 6. As seen in Table 6, results indicate that when T-cells alone, or T-cells + A-cells in the culture system (T-cells + B-cells + A-cells) were exposed to Trasylol (1,000 KIU/culture) for 24, 48 or 72 hr, there was no suppression of the PFC response (680 to 840 PFC/culture) when compared with control cultures (809 $\pm$ 26 PFC/culture). When both B-cells and A-cells in the culture system were exposed to Trasylol for 24, 48 or 72 hr, the plaque number/culture was reduced to a significant degree ($P < .01$). Furthermore, when only B-cells in the culture system of three cell types were exposed for either 24, 48 or 72 hr, there was a significant suppression of the PFC response ($P < .01$). These results clearly demonstrate that suppression brought about by Trasylol was focused on regulation of antigen-dependent B-cell responses.

TABLE 1

Dose-Response Effect of Trasylol on the *In vitro* Primary Antibody Response

Trasylol (KIU/Culture)	Anti-SRBC Responses (PFC/Culture)*
2000	20 ± 4
1000	19 ± 4
500	191 ± 10
250	166 ± 57
100	274 ± 12
HSA (1000 μG)	607 ± 55
---	579 ± 46

Spleen cell cultures of 2×10^7 cells per mL of medium with 0.05 mL of 1.5% SRBC.

*Mean ± SE of five cultures per group.

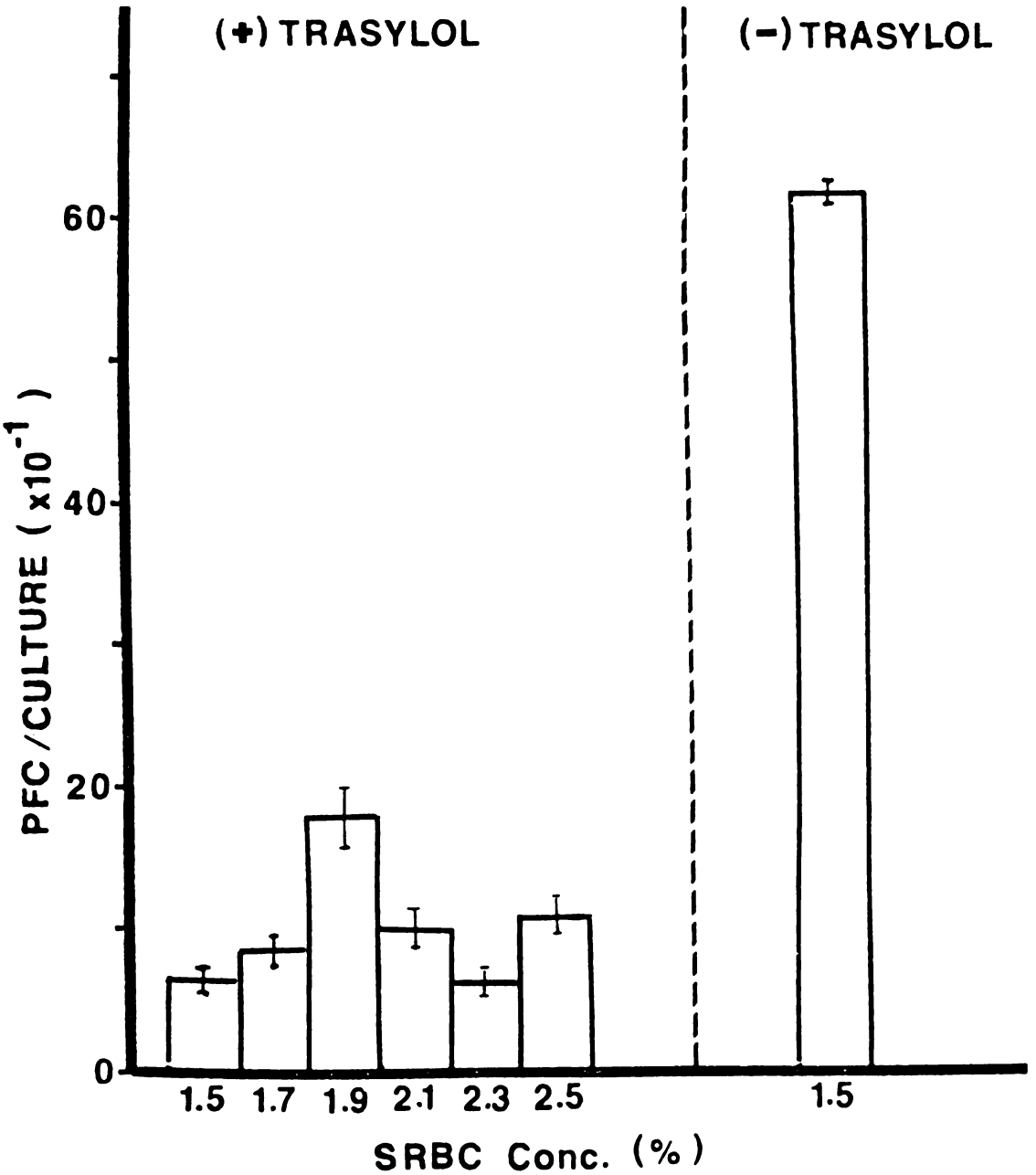

Figure 1. Attempt to override Trasylol suppression by increasing the antigen dose. Spleen cell cultures of 2 x 10^7 cells/ml with varying concentrations of 0.05 ml SRBC. Data are expressed as means $\pm$ standard error of five cultures per group. This represents data from one of two similar experiments.

TABLE 2

TRASYLOL SUPPRESSION OF MEMORY RESPONSE*

TRASYLOL (KIU/CULTURE)	ANTI-SRBC RESPONSES (PFC/CULTURE)‡		
	14 DAYS	21 DAYS	35 DAYS
2000	2666 ± 504	2232 ± 289	2427 ± 372
1000	5890 ± 457	5186 ± 133	4439 ± 163
500	6234 ± 846	5920 ± 204	6300 ± 139
–	8228 ± 561	8753 ± 530	8166 ± 437
NO ANTIGEN	677 ± 77	521 ± 54	443 ± 667

Spleen cell cultures of 2 x 10^7 cells per mL of medium with 0.05 mL of 1.5% SRBC.

*Spleen cells from mice primed with 10^7 SRBC per mouse 14, 21, and 35 days previously.

‡Mean ± SE of five cultures per group.

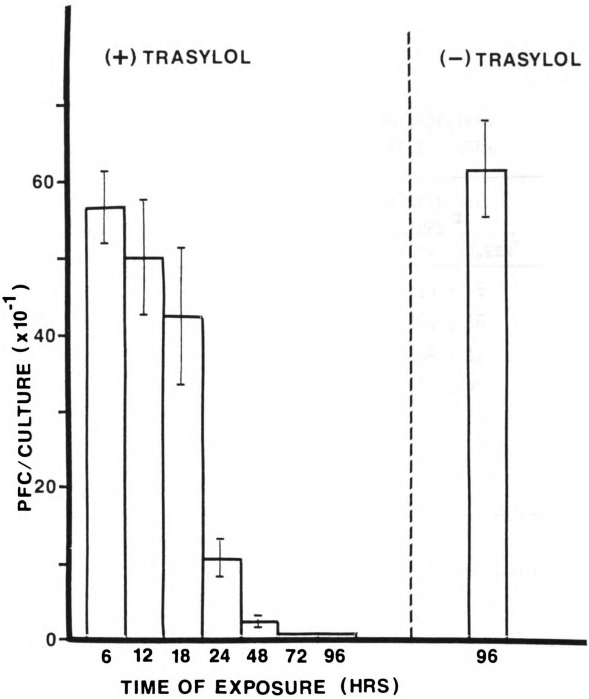

Figure 2. Kinetics of Trasylol suppression on the immune response. Spleen cell cultures of 2 x 10^7 cells/ml and 0.05 ml of 1.5% SRBC. Trasylol was removed from the cultures at varying intervals during the incubation period. Cells were washed three times with fresh medium before added back into the cultures. Data are expressed as means $\pm$ standard error of five cultures per group. This represents data from one of three similar experiments.

TABLE 3

TRASYLOL REGULATION OF THE ANTI-DEXTRAN PFC RESPONSE OF NORMAL AND B-CELL SPLEEN

TRASYLOL (KIU/CULTURE)	ANTI-DEXTRAN RESPONSES (PFC/CULTURE)‡	
	NORMAL SPLEEN	B-CELL SPLEEN*
2000	7 ± 4	18 ± 9
1000	410 ± 48	109 ± 16
500	649 ± 35	194 ± 11
250	957 ± 51	534 ± 40
100	1329 ± 86	744 ± 25
-	1606 ± 121	824 ± 47
- (NO DEXTRAN)	23 ± 9	0 ± 0

Spleen cell cultures of 2×10^7 cells per mL of medium with 0.05 mL of 1.5% SRBC.

*Spleen cells from mice previously thymectomized, X-irradiated, and re-constituted with 1×10^6 bone marrow cells.

‡Mean ± SE of five cultures per group.

TABLE 4

TRYPSIN NEUTRALIZATION OF TRASYLOL IMMUNO-SUPPRESSION*

TRASYLOL (KIU/CULTURE) / TRYPSIN (µG/CULTURE)	ANTI-SRBC RESPONSES (PFC/CULTURE)‡			
	-	500	1000	2000
-	1446 ± 101	537 ± 30	57 ± 12	60 ± 12
50	1740 ± 138	1153 ± 88	851 ± 89	5 ± 1
100	2114 ± 124	1250 ± 65	835 ± 40	13 ± 2
150	1897 ± 129	1835 ± 78	1441 ± 18	45 ± 5

Spleen cell cultures of 2 x 10^7 cells per mL of medium with 0.05 mL of 1.5% SRBC.

* Trasylol pre-incubated for 3 h with varying concentrations of 2x crystallized trypsin.

‡ Mean ± SE of five cultures per group.

TABLE 5

TRASYLOL EFFECT ON ADHERENT AND NON-ADHERENT SPLEEN CELLS FOLLOWING ANTIGEN STIMULATION‡

GROUPS	ANTI-SRBC RESPONSES (PFC/CULTURES)§		
	24 HRS	48	72
A* + NA	712 ± 23	702 ± 20	659 ± 40
A + NA*	120 ± 19	167 ± 18	125 ± 31
A* + NA*			124 ± 19
A + NA			684 ± 38

*Trasylol treatment.

‡Non-adherent cells were separated from adherent cells by repeated washing of cultures in tissue culture dishes with fresh medium.

§ Mean ± SE of five cultures per group.

TABLE 6

The Immunobiological Effects Of Trasylol On T-Cells, B-Cells And Macrophages Of Spleen

Groups‡	Anti-SRBC Responses (PFC/Cultures)§		
	24 hrs	48	72
T* + B + A*	762 ± 36	677 ± 15	692 ± 33
T + B* + A*	27 ± 10	25 ± 8	27 ± 5
T* + B + A	838 ± 15	688 ± 24	722 ± 41
T + B* + A	28 ± 5	27 ± 10	26 ± 2
T* + B* + A*			32 ± 4
T + B + A			809 ± 26
-			796 ± 40

*Trasylol treatment.

‡Cultures in the various groups consisted of 10^7 T cells and 10^7 B cells.

§Mean ± SE of five cultures per group.

DISCUSSION

Our previous observations (18) indicated that α_1-antitrypsin (α_1-AT) a major protease inhibitor present in the normal serum, plays an important role in the regulation of the immune response. Due to difficultire in obtaining large amounts of pure α_1-AT, Trasylol has been used in the present studies as a model for further determining various parameters of immunoregulation brought about by protease inhibitor. Trasylol has a defined chemical composition and its mechanism of interaction with proteases is better understood than for other protease inhibitors. This would facilitate our studies of protease inhibitors in their role as immunoregulators. The present investigation is the first in which Trasylol, a protease inhibitor, has been examined for its effect on the immune response.

Trasylol has been found to be immunosuppressive on both the primary and the secondary antibody production. An equivalent amount of medium or normal human serum albumin (HSA) did not suppress the PFC response. Dose-response studies showed that suppression with Trasylol occurred at concentrations greater than 100 KIU per culture which is equivalent to 14 µg/ml. This amount which has immunosuppressive activity, requires 40-fold less than that found for α_1-antitrypsin (500 µg/ml) which was shown in our previous work to be immunosuppressive (18). Maximal suppression of the 4-day in vitro response to SRBC was achieved by the addition of Trasylol to a final concentration of 2000 KIU per culture.

We confirmed *in vitro* results by injecting varying doses of Trasylol (100-2000 KIU/mouse) and measuring antibody responses to SRBC. Our observations (data unpublished) indicated that Trasylol did not affect the *in vivo* primary antibody response. One possible explanation would be the very short half-life of Trasylol ($T_{\frac{1}{2}}$ = 150 min) *in vivo* which would cause most of the material to be excreted before it exerts an effect on the immune response.

Exposure of the murine splenic cells to suppressive concentrations of Trasylol for nearly 6 h before adding the antigen did not alter their responsiveness. Washing the cells two to three times before the addition of antigen was sufficient to remove all suppressive activity (unpublished observation). Presently studies are under way to determine whether prolonged treatment with Trasylol is needed to alter surface membrane receptors necessary for antigenic recognition or to inhibit intracellular processes required for antibody synthesis.

Results presented above indicate that the immunosuppressive activity of Trasylol could not be reversed by incubating the spleen cell cultures with surplus of antigen. This suggests that immunosuppression was not due to direct interaction or depletion of the antigen.

It is currently thought that normally immunocompetent cells become activated by binding antigen to specific cell receptors. It is possible that Trasylol may inhibit the binding of antigen either by direct association with the antigen receptor or by association with a receptor of its own that sterically prevents the union of antigen with cell surface antibody receptor. Preincubation of spleen cells or the antigen with

suppressive concentrations of Trasylol, before addition into the culture system, have ruled out the above possibility since no suppression resulted in either case. Due to its small molecular size it is unlikely that Trasylol would sterically block the union of antigen with its receptor.

Several other findings bear particular importance in understanding the mechanism of Trasylol regulation of the immune response. A sharp decline of the PFC response occurred after 18 to 24 h when Trasylol was added to spleen cell cultures from zero to 96 h. Shorter exposures (6 to 12 h) resulted in little or no suppression suggesting that the mechanism(s) involved in Trasylol induced suppression was more complex than simply blocking Ig receptors and prevention of antigenic stimulation. The finding that when Trasylol was added to cultures later than 72 h after immunization no effect on the immune response occurred suggesting that the synthetic capacity of the antibody-secreting (B) cell was apparently not affected by Trasylol. However, the early phases of antibody formation such as antigen processing, B-T cell interactions or clonal proliferation of antigen-sensitive cells could be susceptible to the Trasylol-induced suppression. Furthermore, Trasylol addition to spleen cell cultures at various time periods revealed that Trasylol interfered with an early event in lymphocyte stimulation (data unpublished). The critical time for inhibition by Trasylol was 18-24 h in antigen-stimulated spleen cell cultures. These findings coincided well with those reported by Moreau _et al_. (23), who demonstrated that the blastogenesis of human lymphocytes was inhibited by protease inhibitors

such as soybean trypsin inhibitor (SBI) and tosyl-L-lysine chloromethyl ketone (TLCK) which had been added only in an early phase of cultivation.

We have also demonstrated that this immunosuppression can be partially reversed by removal of Trasylol from the culture system and allowing the spleen cell cultures to go for prolonged periods of incubation longer than 4 days (unpublished observations).

A cytotoxic effect of the agent on lymphocytes was ruled out since antibody synthesis by antigen-stimulated spleen cells was not inhibited by pretreatment with Trasylol for nearly 24 h. Also, cell viabilities were unaltered.

The effect of Trasylol on the differentiation of memory cells to antibody secreting plasma cells was also investigated. Trasylol significantly suppressed immunocompetence of splenic lymphocytes in which memory was induced. The fact that Trasylol will suppress antigen-induced proliferation of previously sensitized (SRBC-primed) lymphocytes suggests that it can modify an already established state of immunity. Trasylol may affect the generation of memory cells but this has not been investigated in this study. When SRBC-primed spleen cells were exposed to the same sensitizing antigen, there was a higher secondary PFC response (15-fold higher) compared to the primary _in vitro_ response. Nearly 10-fold more Trasylol was required to suppress the secondary PFC response when compared to the primary. The weak inhibition of the _in vitro_ generation of secondary antibody was in agreement with previous _in vivo_ findings of Nelken (24) where he showed that injection of an

immunosuppressive protein (isolated from normal serum) prior to secondary stimulation produced only a marginal suppressive effect. As most antibodies produced during the secondary response are of the 7s class, the results suggest that both IgM and IgG antibodies are suppressed.

Treatment of Trasylol with an estero-proteinase (trypsin) demonstrated that inhibitory action of Trasylol could be neutralized with this enzyme. Low concentrations of trypsin had no effect since only trypsin, 100-150 μg/ml, preincubated with Trasylol reversed the immunosuppressive effects. The chemical susceptibility of Trasylol parallel the previous work of Viet and Michael (25) and Bullock and Andersson (26,27) who demonstrated that the suppressive activity of normal mouse serum was neutralized by trypsin. Thus Trasylol can be saturated with trypsin, such that the binding of another proteinase, perhaps of cellular nature, is very greatly decreased. Investigations are underway to ascertain that trypsin did not abrogate immunosuppression by enzymatic degradation of Trasylol.

There are at least three different types of cells in mouse spleen required for the antibody response to SRBC *in vitro* (28). At least two of the cell types (T- and B-cells) synergize in producing a response to SRBC (29). These lymphocytes are sensitive to x-irradiation and do not adhere to glass or plastic. The third class of cell type is relatively resistant to irradiation and readily attaches to glass or plastic. Evidence presented here ruled out the possibility that adherent cells are affected by Trasylol. Our findings that pretreatment of T-lymphocytes with Trasylol does not result in any suppression of the PFC

response of spleen cells to SRBC suggest that Trasylol does not affect T-cells. This has been further confirmed by our observation that Trasylol suppressed the immune response to thymus-independent antigens such as dextran sulfate. Thus, our results suggest that the target of the Trasylol inhibition is the B-lymphocyte. Further evidence is presented in a subsequent paper (30) showing that Trasylol suppresses B-cell dependent mitogen-induced lymphocyte transformation. Also, fluorescent labelled Trasylol reacted very intensely with B cells.

Others have found that B-lymphocyte activity can be enhanced by proteases such as trypsin, pronase, elastase-like protease and chymotrypsin-G (31,32). One would assume a natural Trasylol-susceptible protease to be involved in the activation of B-lymphocytes by antigen.

Several laboratories (3,10,33-36) have demonstrated the suppressive actions of protease inhibitors on lymphocyte activation. These agents include TLCK (3,34,35), SBI (23), lima bean inhibitor (37), tosyl-L-arginine methyl ester (3,34), EACA (3), trasylol (10,33), TPCK (3,34), benzoyl-L-arginine amide (34), leupeptin (36) and antipain (38).

None of these inhibitors, however, has been reported to have an inhibitory effect solely on the function of B-cells of spleen. It is of importance to note that only the effect of insolubilized SBI was attributed to an inhibitory action on a protease associated with the lymphocyte surface (23). The protease inhibitors listed above seem to be heterogeneous with respect to the inhibition profile. They include two types of protease inhibitors, i.e., chymo-trypsin-inhibitor and trypsin-inhibitor. It seems likely, therefore, that more than one

protease may be involved in lymphocyte triggering. It is of importance to elucidate how the protease inhibitors under discussion act to inhibit the lymphocyte response.

A shift in the balance between concentrations of protease inhibitors and proteolytic enzymes may condition the degree of lymphocyte differentiation. It is important to consider the possibility that an increase in the concentration of potentially available protease may drive subsets of B-lymphocytes into mitotic cycle and to induce the secretion of antibodies of multiple specificities (39,40) leading to unwanted immunological anomalies. Protease inhibitors, on the other hand, may be important in regulating the level of such proteases.

The demonstration in this and a subsequent study (30) that Trasylol is a potent inhibitor of B-cell function led us to postulate that Trasylol can be used to test the proposal that protease inhibitors play an important immunoregulatory role in lymphocyte differentiation.

SUMMARY

Trasylol (aprotinin), also referred to as kallikrein inactivator, is known to bind and inactivate a variety of estero-proteinases, such as trypsin, chymotrypsin, Cathepsin-G, etc. In concentrations of 100 to 2000 kallikrein inactivating units (KIU) per culture, this inhibitor suppressed both the primary and secondary plaque forming cell (PFC) response of mouse spleen cells to SRBC *in vitro*. Suppression by this kallikrein inactivator was not antigen-specific. This was further verified by the demonstration that Trasylol suppressed *in vitro* PFC response

of spleen cells against dextran (a T-independent antigen). Suppression by Trasylol was not due to depletion effect on the antigen. The inhibitory capacity of Trasylol was reversible. The degree of suppression was dependent on the time at which Trasylol was added to the cultures: Trasylol added to antigen-stimulated cultures up to 48 h after initiation of cultures was immuno-suppressive whereas at 72 h after initiation or later it did not suppress. Pretreatment of spleen cells, with this inhibitor, for approximately 6 h before exposure to the antigen did not affect the immune response. When pre-incubated with trypsin, the suppressive activity of Trasylol was abrogated. Trasylol did not appear to affect T-cells or adherent cells, but it suppressed the B-cell differentiation. These results suggest that Trasylol has an immunoregulatory function.

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ARTICLE 3

PROTEASE INHIBITOR (TRASYLOL) INTERACTION WITH LYMPHOID CELLS[1]

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FOOTNOTES

1. This work was supported by grants from the National Institutes of Health (CA 13396 and AI-12549) and the American Cancer Society (IM-158).

2. H. C. M. is the recipient of an American Cancer Society Faculty Research Award (FRA-147).

3. Abbreviations used in this paper: D-MEM, Dulbecco's modified Eagle's medium containing 10% FCS; FCS, fetal calf serum; PBS, phosphate-buffered saline; HSA, human serum albumin; PFC, plaque-forming cells; LPS, lipopolysaccharide; Con-A, concanavalin-A; (^{3}H) TdR, tritiated-thymidine; TCA, trichloracetic acid; FITC, fluorescein isothiocyanate.

ABSTRACT

By the use of incorporation of radiolabeled thymidine into mouse spleen cells and plasmacytoma (MOPC-21), the inhibitory effect of the protease inhibitor, Trasylol, on mitogen-induced lymphocyte triggering was studied *in vitro*. DNA synthesis was effectively inhibited by 250-1000 kallikrein inactivating units (KIU) of Trasylol when response was induced by lipopolysaccharide endotoxin of *Escherichia coli*. The inhibitory effect of Trasylol was reversible. On the contrary, DNA synthesis of spleen cells was not inhibited by the inhibitor when the cells were stimulated with concanavalin-A. DNA synthesis and growth rate of plasmacytoma (MOPC-21) a B-cell tumor, was also reversibly inhibited by Trasylol. These results suggest that the target of inhibitory action of Trasylol was the B-lymphocyte. Fluorescent labeling studies have also supported these findings since B-lymphocytes and plasmacytoma (a B-cell tumor) display intense binding of labeled Trasylol compared to low levels found on T-cells or lymphoblastoid cell line (S.49.1, a T-cell tumor).

INTRODUCTION

The role of proteolytic enzymes in cellular functions has been expanded in recent years. Proteases have been found on the surface and in the medium of cultured cells (1-3) and it has been suggested that a

proteolytic mechanism may be of importance in the triggering of lymphocytes (4,5). Reports from several laboratories indicate that inhibitors of proteolytic enzymes affect such activities of cells as division (6,7) and the response to plant lectins (8).

In preceding investigations (9,10), we have described the immunoregulatory role of two protease inhibitors. α_1-Antitrypsin and Trasylol based on our observations that both protease inhibitors have immunosuppressive effect on primary and secondary antibody responses in vitro and in vivo. With no outward effect on cell viability, they specifically inhibited the antigen-dependent B-cell differentiation.

The present communication describes the inhibitory action of Trasylol on lymphocyte stimulation by mitogens and gives evidence that this action is exerted only on B-lymphocytes. In this study we have also used fluorescent microscopy to verify that protease inhibitor binds specifically to B-lymphocytes.

MATERIALS AND METHODS

Animals: C57BL/10 x C3H/He (BCF_1) female mice were from Cumberland View Farms, Clinton, Tennessee.

Cells for Culture and Assay: Spleen cells were obtained aseptically from 9- to 16-week old C3H/He x C57BL F_1 female mice (Cumberland View Farms, Tennessee). Cell suspensions were prepared by gentle aspiration with a syringe and needles of progressively increasingly gauge (21 to 27) to obtain a single cell suspension. Spleen cells were washed once and resuspended in medium CMRL 1066 (Grand Island Biological Co., Grand

Island, NY), supplemented with 10% fetal calf serum (FCS) (Grand Island Biological Co.), 0.15 mM L-asparagine, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 mg/l gentamycin. Spleen cells were cultured in 96 well Falcon plastic flat-bottomed tissue culture plates (Falcon Plastics, Div. of BioQuest, Oxnard, CA). Viability of cells was determined by trypan blue exclusion in all experiments.

Trasylol: Trypsin-kallikrein Inhibitor (Aprotinin) (FBA Pharmaceuticals, New York, NY) was diluted in spleen cell medium (pH 7.2-7.4) to obtain a desired concentration/culture.

Stimulants:

Lipopolysaccharide: Eschericia coli 0127:B8 Lipopolysaccharide (LPS) (Difco Laboratories, Detroit, Michigan) was dissolved in PBS such that each 1.0 ml contained 100 µg. The LPS suspension was boiled for 30 minutes, and filter sterilized prior to use. From the stock LPS solution, dilutions were made in the spleen cell culture medium such that a 0.05 ml volume contained the appropriate amount of LPS.

Concanavalin-A: Purified Concanavalin-A (Con-A) was purchased from Difco Laboratories, Detroit, Michigan. The Con-A was dissolved in 0.01 M phosphate buffered saline (PBS, pH 7.2) immediately before use and filter sterilized. Dilutions of the stock Con-A solution were made in spleen cell culture medium such that a 0.05 volume contained the appropriate amount of Con-A.

Mitogen Reactivity: Cultured BC_3F_1 spleen cells were assayed for reactivity to the mitogens Concanavalin-A (Con-A) and lipopolysaccharide (LPS) using a microculture system. Spleen cell suspensions of 5×10^5

viable cells (viability greater than 85%) in 0.1 ml of spleen cell culture medium and 0.05 ml of the mitogen were cultured in 96 wells Falcon Plastic flat-bottomed tissue culture plates (Falcon Plastics, Div. of BioQuest, Oxnard, CA). The cell cultures were set up in triplicate and incubated in a humidified atmosphere of 8% CO_2 and 92% air for different time periods ranging from 24 to 96 hr.

The experiments were performed using 0.05 ml of Con-A (20 µg/ml), LPS (100 µg/ml) or medium only. Trasylol was added at the beginning of cultures, so that 0.05 ml contains the desired concentration/well.

Plasmacytoma and Lymphoblastoid cells: The myeloma cell line Mineral Oil Plasmacytoma 21 (MPOC-21) used in this study was provided by Dr. Ronald J. Patterson (Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan). Murine lymphoblastois cell line S.49.1 (BALB/c, H-2^d, Thy-1.2) (11) was obtained from the Salk Institute Cell Distribution Center (La Jolla, California). These cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) (Grand Island Biological Co., Grand Island, New York) with 10% heat inactivated FCS (D-MEM/FCS) supplemented with 3.5 g dextrose/1, 3.7 g $NaHCO_3$/1 and penicillin and mycostatin (each agent 100,000 units/1) and streptomycin (100,000 µg/1). Cell viabilities were determined by trypsin blue exclusion in all experiments.

Trasylol Effect on Cell Multiplication: To examine the effect of protease inhibitor, Trasylol, on the multiplication of these two cell lines, plasmacytoma (MOPC-21), and lymphoblastoic (S.49.1) cell lines were cultured in D-MEM/FCS medium for time periods ranging from 6-96 h.

These cell lines were prepared in suspensions of 2 x 10^5 viable cells/ml (viabilities greater than 98%) in 50 ml of D-MEM/FCS and cultured in flasks (Falcon Plastics, 3024, 75, cm^2, Oxnard, California) at 37°C in humid 8% CO_2 atmosphere. Replicate culture flasks were prepared for each designated period of incubation. Trasylol to be tested for its suppression of growth of these cell lines, was added in concentrations ranging from 250 KIU to 1,000 KIU/ml of culture at the beginning of culture unless otherwise stated.

Thymidine Incorporation and Uptake: After an initial 24, 48, 72 and 96 h of culturing, the cells were pulsed with 1.0 µCi of ^{3}H-thymidine, (^{3}H)TdR (Specific Activity 2.0 Ci/mmole, New England Nuclear, Boston, Mass.), for the last 6 hr of culture. The cultures were harvested for counting by collecting the cells on Whatman 934 AH (Whatman Inc., Clifton, NU) glass fiber filters. The cells were washed with phosphate buffered saline (PBS) pH 7.2-7.4, using a microculture automated harvesting device MASH II harvester (Microbiological Associates, Klalleersville, Maryland). The glass filters were dried and then placed in glass scintillation vials containing 5 ml of scintillation fluid (Toluene base-Omnifluor, NEN, Boston, Mass.). The samples were counted for 5 min after allowing for equilibration using a Searle Delta-300 (Searle Analytic Inc.) liquid scintillation counter, model #6890.

In experiments involving the effect of Trasylol on plasmacytoma and lymphoblastoid cell lines, 1 ml aliquots of cells were collected in triplicate in test tubes. The cells of both control and Trasylol treated groups were pulsed with 2.0 µCi of (^{3}H)TdR for 30 min at 37°C.

These were then lysed using 5% sodium dodecyl sulfate (SDS), followed by precipitation with 50% trichloracetic acid (TCA). The TCA precipitates were collected on filter membranes and their radioactivity counted as described previously.

The arithmetic mean of the triplicate samples was determined and the results are expressed as the mean counts per minute (cpm) ± S.E. (standard error of the mean).

Viable Growth Studies: Both plasmacytoma (MPOC-21) and lymphoblastoid cell line (S.49.1) were cultured with various concentrations of Trasylol ranging from 250 to 1,000 KIU/ml. The control flasks received only an equivalent amount of medium. At the end of every 6 h, aliquots were collected and viable cell counts were made of cell lines in both the Trasylol-treated and untreated groups. Results are expressed as the mean viable cell count of triplicate determinations ± S.E.

Fluorescent Staining Method:

a. Reaction of Trasylol with Fluorexcein Isothiocyamate:

To a 2.0 ml Trasylol solution (1.4 mg/ml) was added 0.5 ml of carbonate-bicarbonate buffer followed by slow addition of 2.5 ml of FITC solution. The mixture was then left on the rocker for 1-2 h at room temperature. The fluorescein-conjugated Trasylol was separated from the unreacted FITC by passage through Sephadex G-10 (Sigma Chemical Co., St. Louis, MO) column.

b. Fluorescent Labeling of Cells:

Thin smears of cells were prepared on pre-cleaned, albumin coated slides. The cell smears were methanol fixed, air dried, and kept in

0.1 M PBS at room temperature for 1-2 h. The fixed cell smears were reacted with fluorescein-conjugated Trasylol for 30 min at 37½C in 100% moisture. For control, the cell smears were treated with fluorescein-conjugated normal human serum albumin (HSA), or an unreacted FITC. After washing the cells free of unbound protein, they were air dried, mounted under a cover slip in buffered glycerol (Glycerol:PBS-9:1) and then examined by fluorescent optics.

c. Inhibition of Fluorescent Labeling of Cells:

All cell types were first treated in suspension or as a smear, with a non-conjugated Trasylol in PBS for 30 min at 37°C. After washing the Trasylol-treated and untreated controls, cell smears were stained with fluorescein-conjugated Trasylol as described previously.

d. Microscopy:

Fluorescence observations were made with Zeiss Photomicroscope G40-430 by using a x40 high dry Neofluor lens and an epi-illuminator. Fluorescein-fluorescence was excited with a super pressure mercury lamp HBO 200 W, and the filter combinations BG 12 (thickness, 3 mm) and BF 004100, respectively, were used for observations. Photography was performed with Kodak (Ektachrome, 100 Tungsten) filter using the Leica (M1) camera.

RESULTS

Effect of Trasylol on Mitogen Reactivity of Normal Spleen Cells to LPS:

B-lymphocyte mitogen reactivity was used to test the effects of a protease inhibitor, Trasylol. Figure 1 shows the effect of adding

different concentrations of Trasylol to cultures stimulated with 5 μg of LPS (endotoxin or lipopolysaccharide of Gram-negative bacteria) under standard culturing conditions. When increasing concentrations of Trasylol 250 KIU to 1,000 KIU/culture were added to the cultures, there was a concommitant inhibition of mitogenicity reflected by a decrease in radiolabeled thymidine [(^{3}H)-thymidine] incorporation. Inhibition of LPS mitogenicity by 500 and 1,000 KIU of Trasylol at 24 h was significant at $P < .05$ (Figure 1a). When incorporation was measured 48 hr after addition of LPS, 250 KIU of Trasylol/ml inhibited an average of 42% of ^{3}H-thymidine incorporation (Figure 1b). At this time period, higher concentrations of Trasylol (500 and 1,000 KIU/ml) reduced the LPS-induced incorporation of ^{3}H-thymidine by over 90%. For example, when stimulation of lymphocytes by LPS resulted in an incorporation of 4.423 $\pm$ 139 cpm, compared to an incorporation of 688 $\pm$ 44 cpm by non-stimulated spleen cells, the addition of Trasylol (500 and 1,000 KIU/ml) at 0 h to LPS stimulated cultures resulted in the ^{3}H-thymidine incorporation of only 1,788 $\pm$ 29 and 254 $\pm$ 93 cpm, respectively at 48 h.

Trasylol did not suppress the amount of ^{3}H-thymidine incorporation by unstimulated cells which demonstrated an absence of cytotoxicity of Trasylol for the cultured lymphocytes. This was consistent with our previous study (10) in which Trasylol had no significant effect on cell viability of cultured lymphocytes. By 72 and 96 h, the ^{3}H-thymidine incorporation by LPS stimulated cultures of spleen, was reduced significantly ($P < .001$) at all three concentrations (250, 500 and 1,000 KIU/ml) of Trasylol used in the studies (Figures 1c and 1d).

Effect of Trasylol on Mitogen Reactivity of Normal Spleen Cells to Con-A:

As a test of T-cell reactivity, Trasylol was further examined for its effect on whole spleen cells when they were stimulated with 1 µg/culture of Concanavalin-A (Con-A, a T-cell mitogen). Results indicated (data not presented) that with 500 KIU of Trasylol/culture, there was no effect in the ^{3}H-thymidine incorporation by spleen cells stimulated by Con-A even after 96 h of culturing. With a 1,000 KIU of Trasylol, only a slight decrease of ^{3}H-thymidine incorporation by Con-A stimulated spleen cells occurred, but this suppression was not significant at 96 h of exposure.

These and the previous results with LPS-stimulation suggested that the inhibitory action of Trasylol on lymphocyte response might be related to its effect on B-cells. Furthermore, the amount of Trasylol effective for depression (500 and 1,000 KIU/ml) of LPS stimulation was very close to that effective for suppression of antigen responses (10).

Reversal of Trasylol Suppression of Mitogen Reactivity of Spleen Cells to LPS:

It was possible that a subtle impairment of the cellular function interfered with the cell's ability to increase the rate of ^{3}H-thymidine incorporation in response to LPS. Therefore, cells were pre-incubated at 37^{o}C with varying concentrations of Trasylol (250 to 1,000 KIU) for 24 h. Cells were also simply incubated as a control. At the end of 24 h the cells were washed twice with [D-MEM-(FCS)] to remove Trasylol and their capacity to respond to LPS was measured. This was done by adding LPS and measuring ^{3}H-thymidine incorporation from 24 to 96 h after

addition of LPS. As a positive control the capacity of the pre-incubated washed cells to be inhibited by Trasylol was also measured. Thus, after incubating cells for 24 h in medium alone and washing them, Trasylol was added to those cells simultaneously with the addition of LPS. Cells incubated with Trasylol and LPS simultaneously showed the expected reduction of ^{3}H-thymidine incorporation at 500 and 1,000 KIU of Trasylol. In contrast, cells treated with the same concentration of Trasylol for an identical length of time followed by stimulation with LPS, incorporated ^{3}H-thymidine as well as by cells treated with LPS alone (data not presented). Since pre-incubation of cells with Trasylol did not affect their subsequent ability to respond to LPS it appeared that Trasylol did not irreversibly or non-specifically damage cells. Furthermore, since the effect of Trasylol could be abolished by simple washing procedures, it appeared likely that the inhibitor was not tightly bound within the cell.

Suppression of Mouse Plasmacytoma (MOPC-21) Growth by Trasylol:

In our previous biological observations, the inhibitory action of Trasylol on the plaque forming cell (PFC) response of spleen cells against SRBC (10) and on mitogenic stimulation of spleen cells (Figures 1a-d) was directed only to B-lymphocytes. Whether the effect of LPS on mouse lymphocytes is exclusively on B-cells as originally reported (12) or possibly on T-cells remains entirely unclear. Recent data (13) suggest that the mitogenic effect of LPS on B-cells may require the participation of T-cells. It is thus difficult to be certain at this

time if the suppressive effect of Trasylol on LPS stimulation is due to a direct effect of Trasylol on B-cells or whether it is acting via a T-cell. This led us to confirm our observations by studying the effect of Trasylol on the growth of plasmacytoma (MOPC-21) cell line, which is a B-cell tumor. For this MOPC-21 cultures were exposed to varying concentrations (500 and 1,000 KIU/ml) of Trasylol. Control cultures received only the medium. At the end of incubation period of every 6 h, an aliquot was taken from each group and viable cell counts in triplicate were made by using trypan blue exclusion method.

As shown in Figure 2, the results indicated that there was no increase in the number of viable cells in cultures containing 1,000 KIU/ml of Trasylol. Cultures with 500 KIU of Trasylol/ml exhibited a slower increase in the cell number with a doubling time of approximately 60 h, compared to the control cultures where the cell number doubled within 24 h of incubation. These results, therefore, suggest that 500 KIU of Trasylol/ml suppresses the viable cell growth of B-cell line (MOPC-21).

DNA synthesis by plasmacytoma (MOPC-21) was measured in the presence of Trasylol. Plasmacytoma cell line was cultured with 500 and 1,000 KIU of Trasylol/ml, respectively. Control cultures received only the medium. An aliquot was obtained every 6 h of incubation, and DNA synthesis by MOPC-21 was determined as a measure of their ability to incorporate ^{3}H-thymidine. As shown in Figure 3 there was a gradual increase of DNA synthesis in cultures within 36 h of incubation. Both concentrations of Trasylol (500 and 1,000 KIU/ml) suppressed DNA synthesis of MOPC-21, e.g., at the peak of DNA synthesis, at 36 h of incubation,

cultures with 500 and 1,000 LIU of Trasylol/ml incorporated 130,711 ± 7,507 (cpm) and 63,648 ± 1,354 (cpm) respectively of ^{3}H-thymidine compared to the control cultures which incorporated 187,732 ± 7,900 (cpm) of ^{3}H-thymidine during the same time period.

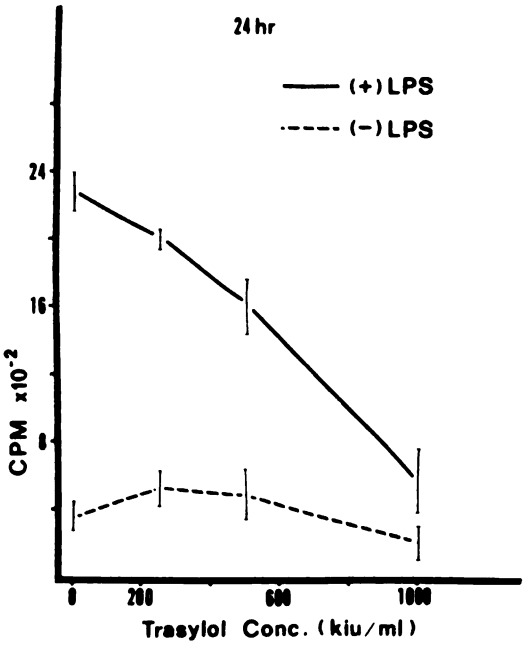
Reversal of Trasylol Suppression Upon its Removal from the Cultures: To see whether Trasylol effect is reversible, plasmacytoma cell line (MOPC-21) was incubated with varying concentration (500 and k,000 KIU) of Trasylol. The control cultures received only the medium. After 24 h of incubation, Trasylol was washed away from the cultures and viable cell counts were measured by trypan blue dye exclusion method, every 6 h of incubation period up to 60 h. We observed that the doubling time ($T_{\frac{1}{2}}$) of Trasylol-pretreated cultures was similar to that of the control cultures ($T_{\frac{1}{2}}$ = 16-18 h). These results (data not presented) therefore indicated that Trasylol does not irreversibly damage cells.

Fluorescent Labeling of Lymphoid Cells: In this study we have used fluorescein-labeled protease inhibitor to directly visualize various cell types. The results confirm the interpretation based on immunological and mitogen-stimulation methods. The normal unstained cells of the spleen exhibit very little or no fluorescence. When fixed normal spleen cells were stained directly, using fluorescein-Trasylol conjugate, fluorescence could be seen over the entire cell surface as well as within the cell (Figure 4a). No fluorescence was seen when the normal spleen cells were pre-treated with Trasylol and stained in a similar manner (Figure 4b). When relatively pure populations of B-cells (Figure 4c) and T-cells (Figure 4d) were examined by staining with

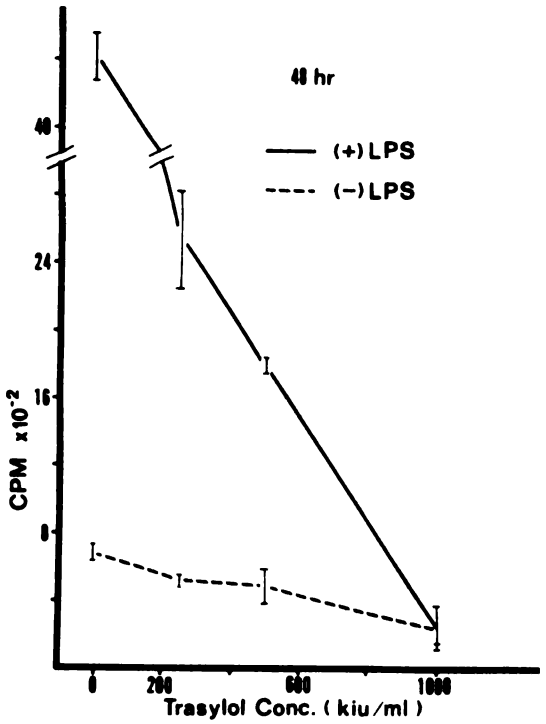
Fluorescein-Trasylol conjugate, B-cells showed staining predominantly at the cell membrane with areas of weak diffuse cytoplasmic fluorescence. In contrast, T-cells showed relatively little or no fluorescence. Cells stained with fluorescein-human serum albumin (HSA) conjugate were negative.

A similar intense staining (both of cell membrane and diffuse cytoplasm) was seen in plasmacytoma (MOPC-21) a pure BOcell line (Figure 4e). Once again, when lymphoblastoid cells (S.49.1, a T-cell line) were stained in a similar manner (Figure 4f) cells showed fluorescence comparable to that of the background controls.

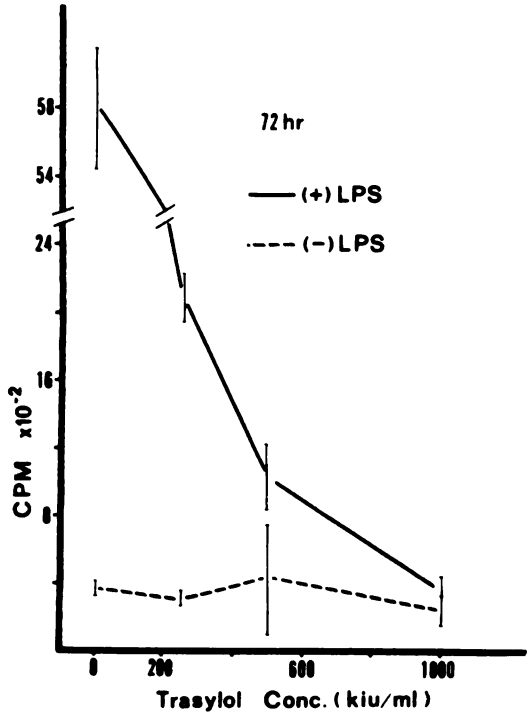
Figure 1. Suppression of LPS-stimulation of spleen cells by various concentrations of Trasylol [250, 500, and 1000 KIU/ml]. ^{3}H-thymidine incorporation by whole spleen cells in the presence (———) or absence (-----) of LPS was measured at various intervals during the incubation period. (a) 24 hr, (b) 48 hr, (c) 72 hr, and (d) 96 hr. Data are expressed as mean counts per minute $\pm$ standard error [cpm $\pm$ SE] of three cultures.



(a)



(b)



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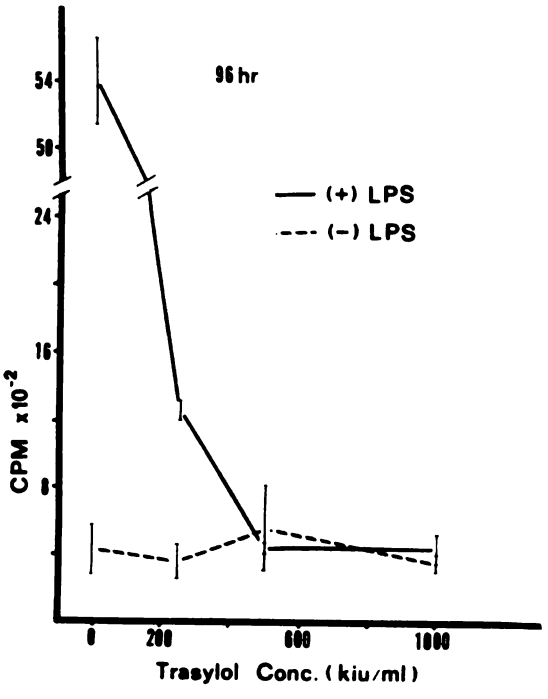


(d)

Figure 1

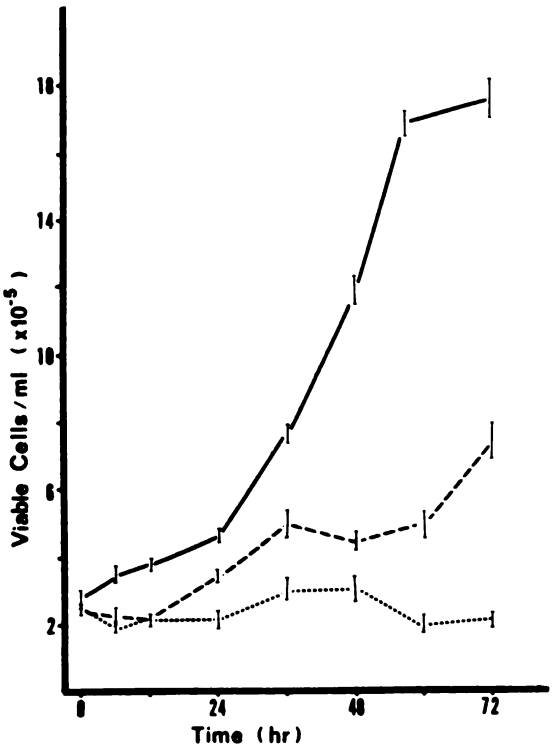


Figure 2. Suppression of mouse plasmacytoma [MOPC-21] growth by Trasylol. Viable plasmacytoma cells/ml were counted at various intervals during the incubation period, in the presence of medium alone (———) 500 KIU/ml of Trasylol (— — — —) and 1000 KIU/ml of Trasylol (-----). Data are expressed as mean viable count ± standard error.

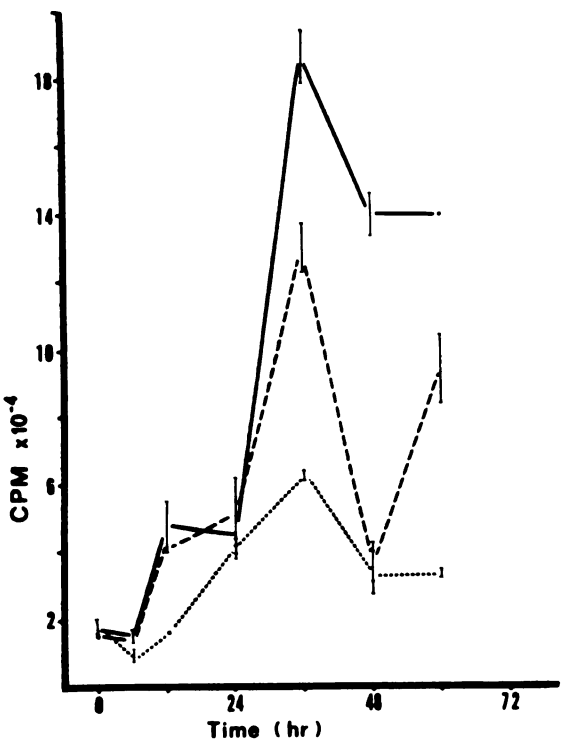
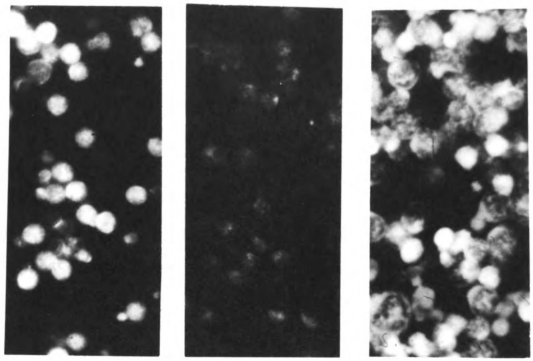
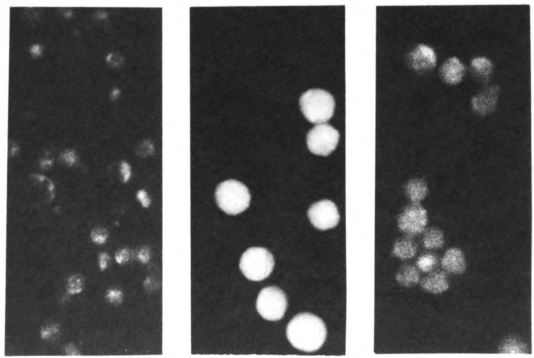


Figure 3. Suppression of mouse plasmacytoma [MOPC-21] growth by Trasylol. ^{3}H-thymidine incorporation by plasmacytoma cell DNA was measured at various intervals during the incubation period, in the presence of medium alone (———), 500 KIU/ml of Trasylol (– – – –), and 1000 KIU/ml of Trasylol (-----). Data are expressed as mean counts per minute ± standard error [cpm ± SE].

Figure 4. Fixed normal spleen cells (a) and Trasylol treated spleen cells (b) stained with FITC-Trasylol. Enriched population of B-cells (c) and T-cells (d) stained with FITC-Trasylol. Plasmacytoma [MOPC-21] cells (e) and lymphoblastoid [S.49.1] cells (f) stained with FITC-Trasylol. Magnification $\approx$ X1800.



(a) (b) (c)



(d) (e) (f)

Figure - 4

DISCUSSION

In the present investigation it was demonstrated that Trasylol in concentrations comparable to those we have shown to suppress antibody synthesis (10), effectively inhibits normal splenic lymphocyte stimulation by a B-cell mitogen (LPS), but not by T-cell mitogen (Con-A) as revealed by ^{3}H-thymidine incorporation. Dose-response studies demonstrated that Trasylol was suppressive at 250-1000 KIU/culture. The only exception was DNA synthesis of spleen cells stimulated with Con-A. The Con-A induced response of spleen cells was not inhibited by Trasylol at doses up to 1000 KIU/culture (unpublished observations). Thus only B-lymphocytes appeared to be susceptible to the inhibitory effect of Trasylol. The mechanism involved in the inhibition by Trasylol of the LPS-induced response is unclear and is under investigation.

A number of obvious causes have to be considered before interpreting the significance of inhibition. First, apparent inhibition of transformation might simply reflect a killing of the responding cell. We have examined this possibility for Trasylol and have found no gross signs of toxicity as reflected by alterations in cell viability, as measured by dye exclusion or by alterations in cell number. However, changes in these parameters require either actual cell lysis or a major alteration in cellular permeability in order to be detected. Therefore, a means of detecting a more subtle, but permanent interference with cellular function was sought. The capacity of these cells to respond to LPS after pre-incubation with Trasylol was found to be unimpaired,

indicating that no permanent non-specific cell damage had occurred. Furthermore, the inhibition by Trasylol of the spleen cell response to LPS was reversible; that is, removal of Trasylol by washing the cells after 24 h of cultivation allowed DNA synthesis and growth rate to resume (data not presented).

Secondly, inhibition could simply reflect a block in some step specific to the incorporation of the precursor being used to measure macromolecular synthesis as a function of transformation. This was considered unlikely since DNA synthesis by B-cells could be inhibited by Trasylol; whereas, DNA synthesis by Con-A stimulated spleen cells was not inhibited by the presence of Trasylol throughout the period of cultivation indicating that inhibition of LPS-induced stimulation observed with Trasylol was not due to a block in the incorporation of the precursor being used to measure DNA synthesis.

Plasmacytoma (MOPC-21) is a homogeneous, albeit more mature, neoplastic B-cell line compared to a heterogeneous population of spleen cells previously examined by us. It is also a self-multiplying tumor in vitro and there is no need for any stimulating agent. We, therefore, were eager to test the effect of protease inhibitor on this cell type.

Trasylol strongly inhibited the growth of plasmacytoma cell line as revealed by a decrease in the incorporation of ^{3}H-thymidine into DNA. The growth rate or the generation time of plasmacytoma with Trasylol was definitely longer than those cultures of plasmacytoma that were not treated with Trasylol. Trasylol produced a steady reduction in cell growth. Our results thus suggest that protease inhibitor, Trasylol,

can modify both the DNA synthesis and the rate of growth of cultured tumor cells.

The inhibition of plasmacytoma cell growth observed, when Trasylol was added to the culture medium, would appear to be the reverse of the effect seen when trypsin is added to a confluent sheet of cells (14,15), or when overgrowth factor was added to a crowded culture (16). Once again, inhibition by Trasylol of plasmacytoma growth was reversible, that is, removal of Trasylol by washing the cells after 24 h of cultivation allowed plasmacytoma to resume normal growth pattern. These observations suggest that there is no apparent permanent cell damage following Trasylol treatment. Hirschhorn et al. (8) have suggested that ε-amino caproic acid (EACA), as well as tosyl-L-lysine chloromethyl ketone (TLCK) and tosylamide-2-phenylethylchloromethyl ketone (TPCK), might inhibit a proteolytic pathway related to the template activity of the lymphocyte nucleus. Reports from several laboratories (8,17-22) have demonstrated the inhibitory actions of protease inhibitors on lymphocyte activation. A list of protease inhibitors reported to be effective for inhibition of lymphocyte responses include TLCK (8,20,21), SBI (19), lima bean inhibitor (5), tosyl-L-arginine methyl ester (8,20), EACA (8), Trasylol (17,18,23) TPCK (8,20), benzoyl-L-arginine amide (20), and leupeptin (22). None of these inhibitors was reported to have an inhibitory effect on the function of B-cells.

Cytophotometry has proven to be a valuable tool in immunology. In this way weak reactions can be detected in reproductable system. In consecutive pilot assays we used fluorescence microscopy. FITC-conjugated Trasylol reacted specifically with the assumed protease on the

cells. Results indicated that fluorescein-conjugated Trasylol bound to certain cells of the spleen. When presaturated with unconjugated Trasylol, these did not show any binding of conjugated Trasylol, suggesting that specific binding took place. B-cells exhibited intense binding of FITC-conjugated Trasylol when compared to T-cells. Similarly, the plasmacytoma cell line (a B-cell tumor) showed intense fluorescent staining with FITC-conjugated Trasylol, while lymphoblastoid cell lines (S.49.1, a T-cell tumor) exhibited little or no binding of FITC-conjugated Trasylol. This fluorescent labeling approach confirms our previous observations (10) that Trasylol binds to and regulate the functions of B-lymphocytes.

We have entertained the possibility that proteolysis brings about alterations in macromolecular synthesis accompanying LPS-stimulation of B-lymphocytes and that Trasylol acts by inhibiting such proteolysis. Consideration of the hypothesis has been stimulated both by our own findings and by some of the current views as to the possible mechanisms of transcriptional controls (8). Previous findings must be considered when evaluating any mechanism for lymphocyte stimulation or its inhibition. It appears that binding of LPS to the cell membrane is the initiating factor in lymphocyte stimulation (24) provided this binding is sufficient to activate the lymphocyte, although no attempt was made in this study to examine this parameter.

A proteolytic system, could provide an amplifying mechanism to produce all of the previously described early alterations after lymphocyte stimulation. It remains to be demonstrated which protease system

exists in lymphocytes, at what stages of differentiation activation takes place and, more precisely, at what cell phase they are functional. The available evidence suggests inhibition of proteolysis during early stages of differentiation to be the most tenable explanation for the observed inhibition of B-lymphocyte transformation by protease inhibitor.

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