CHANGES IN IMMUNE RESPONSE WITH T-2 TOXIN ON SPLENIC LYMPHOCYTES

Dissertation Submitted to the Jawaharlal Nehru University
in Partial Fulfilment of the Requirements
for the Award of the Degree of
MASTER OF PHILOSOPHY

CHINNAM PRASAD RAO

SCHOOL OF ENVIRONMENTAL SCIENCES
JAWAHARLAL NEHRU UNIVERISTY
NEW DELHI - 110067
INDIA
1992

DEDICATED TO THE PERENNIAL FLOW OF

LOVE AND COMPASSION OF

MOTHER TERESA

जवाहरलाल नेहरु विश्वविद्यालय
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI - 110067

SCHOOL OF ENVIRONMENTAL SCIENCES

May 1992

CERTIFICATE

The research work entitled "CHANGES IN IMMUNE RESPONSE WITH T-2 TOXIN ON SPLENIC LYMPHOCYTES as" embodied in this dissertation has been carried out in the School of Environmental Sciences, Jawharlal Nehru University, New Delhi. This work is original and has not been submitted so far in part or full for any other degree or diploma of any University.

Ch. Prasad Rao 9|6|1992
CHINNAM PRASAD RAO
(Candiate)

Prof. V. SUBRAMANIAN
(Dean)
2|7|12

Dr. (Mrs.) JAYASHRI DEVI SHARMA 20|7|92
(Supervisor)

ACKNOWLEDGEMENTS

<div align="right">Ch. Prasad Rao</div>

II

ABBREVIATIONS

Ac DON		Acetyldeoxynivalenol
C^o	:	Centigrade
Con A	:	Concannavaline A
CPM	:	Count Per Minute
DNA	:	Deoxyribonucleic Acid
DON	:	Deoxynivalenol
FCS	:	Foetal Calf Serum
G	:	Gram
GSH	:	Glutathione
NIV	:	Nivalenol
MHC	:	Major Histocompatibility Complex
PBS	:	Phosphate Buffer Saline
PHA	:	Phytohemaglutinin
Popop	:	1, 4 Bis (5 phenyl oxazol 241) benzene
PPO	:	2, 5 Diphenyl oxazolyl
PWM	:	Poak Weed Mitogen
RNA	:	Ribonucleic Acid
RPMI	:	Rosewell Park Memorial Institute
S.D	:	Standard Deviation
SRBC	:	Sheep Red Blood Cell
T-2	:	4 β-15-Diacetoxy-3α Hydroxy-8-α - [3 -methylbutyryloxy]-12, 13 epoxytrichothec-9-ene
U	:	Unit
μci	:	Microcuri

CONTENTS

INTRODUCTION

Mycotoxins are secondary metabolites, produced by various fungi. The most important mycotoxin producing fungi are <u>Pencilium</u>, <u>Fusarium</u>, <u>Cladosporium</u>, <u>Aspergillus</u>, <u>Trichoderma</u>. The most important mycotoxins are Aflatoxins, **Trichothecenes** which include **T-2 Toxin**, Ochratoxin, Patulin. Mycotoxin production is favoured by high humidity and high water activity.

<u>Trichothecene mycotoxins</u> are sesquiterpenoid metabolites produced by several groups of filamentous Fungi. The most investigated Trichothecene mycotoxin is T-2 Toxin. which is produced by <u>Fusarium sporotrichioides</u>.

T-2 Toxin is a major Trichothecene mycotoxin often involved in causing serious losses to agriculture in terms of crop production, and food contamination. The compound is a <u>natural contaminant</u> of cereal grains and has been associated with widely prevalent outbreaks of Toxicosis in animals and Human beings, e.g. in Russia (1942-47), Finland (1982-84), and India (1987). Human exposure to T-2 has also occured as a result of Biochemical warfare and is evidenced by the detection of this Toxin in samples collected in the warzone of South East Asia.

Contamination of this toxin in various food results in a variety of diseases, of which most important is

1

Alimentary toxic Aleukia (ATA) and Stachybotriotoxicosis,
affecting mucosa and immune systems. Leucopenia, anemia,
Bone marrow aplasia, haemorrhagic diathesis are important
pathological symptoms of these diseases in both animals and
humans. T-2 Toxin has been known to cause severe haemato-
poietic damage, as one of the principal features of ATA in
man. T-2 Toxin has also been observed as potent
immunosuppressive agent , affecting the Lymphocytes

Lymphocyte is unique, as it is the only cell that is
highly differentiated to perform one of the most vital
functions of the body. During this immune response, it
becomes large in size, builds up all the necessary synthetic
machinery, to start synthesizing protein, lipid, RNA and
DNA. Simultaneoulsy it acquires the capabilities of per-
forming its functions of defense preparedness for the anti-
genic offensive attack on its targets. This awakening of
lymphocytes is variously called as **lymphocyte transfor-
mation, lymphocyte activation, lymphocyte blastogenesis.**

Physiologically lymphocyte proliferation must occur
only when the specefic clones of lymphocytes encounter the
foreign antigens in vivo. Culture T and B lymphocytes can be
induced to proliferate in vitro by specific stimuli
including specific antigens and allogenic cells.
Unfortunately, mechanistic studies on the initiation of
proliferation are rendered difficult because very small
percentage of lymphocytes actually respond to specific

2

antigens. They are generally not detectable within the limits of accuracy of standard Biochemical methods.

The studies on proliferation of lymphocytes has been fortunately overcome by the use of mitogens which stimulate 70 to 80% of the lymphocytes to proliferate irrespective of their antigen specificity. Though mitogens are diverse in their chemical nature. Most of them are derived from the plant lectins - a group of carbohydrate-binding proteins (eg. Con-A, PHA, PWM etc.) and Bacterial polysaccharides (eg. LPS).

T-2 Toxin was shown to inhibit rapidly dividing cells of the haemopoietic tissue as well as those of the gastrointestinal tract. It has been shown to suppress the lymphocyte response to T and B cell mitogens.

The extreme sensitivity of lymphoid tissue to T-2 Toxin may also be explained by the fact that these cells posses only low level of detoxification enzymes such as Glutathion transferase or there may be receptor for this toxin on the surface of the cells. Several lymphocytic functions such as lymphokinin production, antibody response to T-dependent antigen, have shown to be severely impaired after in vivo treatment with T-2 Toxin. The reduction in number of lymphocytes may be a primary cause of impaired immunity. The immunosuppressive effect may partly be explained by the ability of T-2 Toxin to inhibit DNA, RNA

and protein synthesis in con-A, PHA, poakweed mitogen stimulated lymphocytes. When administered in vivo it decreased serum immunoglobulin and suppressed antibody synthesis against SRBC.

This study is concerned with the effect of T-2 Toxin on cell-mediated immunity. Among the various in vitro methods available, for assaying cell mediated immunity, the lymphocyte transformation test is one of the widely accepted tools. The present study is an attempt to find out whether T-2 Toxin can cause any impairment to the normal immologic response in vivo using in vitro lymphocyte transformation as a tool.

REVIEW OF LITERATURE

1. Mycotoxin Producing Fungi

2. Mycroflora of Various Food Stuffs

3. Source of Food Contamination

4. Occurrence of Mycotoxins

5. Detection and Identification

    5.1 Physical Methods
    5.2 Chemical Methods
    5.3 Immunoassay Methods

6. Mycotoxin Biosynthesis

    6.1 Mycotoxins Derived from Amino Acids
    6.2 Mycotoxins Derived from the Aromatic
         Biosynthetic Pathway
    6.3 Mycotoxins Derived from the Acetate Mevalonate
         Pathway

7. Some Aspects of Structure and Activity Relationship
    in Mycotoxins

8. Trichothecene Mycotoxins

9. T-2 Toxin

    9.1 Precursors of T-2 Toxin
    9.2 Chemical Nature of T-2 Toxin

10. Regulation and Control

11. Use and Applications

12. T-2 Toxin and Biological Response

    12.i General Toxicity
    12.2 The Haematopoietic System with T-2 Toxin
    12.3 Enzymatic Changes with T-2 Toxin
    12.4 T-2 Toxin Metabolism and its Detoxification

13. Some Immunological Aspects with T-2 Toxin

    13.1 Humoral Response and Antibody Synthesis with T-2
          Toxin
    13.2 Skin Response with T-2 Toxin
    13.3 Lymphocyte Response and Macromolecular Synthesis
          with T-2 Toxin
    13.4 Effect of T-2 Toxin on Lymphocyte Proliferation

5

1.Mycotoxin Producing Fungi

Aflotoxin and Sterigmatocystin Producing Fungi

Aspergillus flavus var. columnaris and A. parasiticus were first recognised as aflotoxin producing fungi. Later several investigators have studied the aflatoxin producing ability of other fungal species like A. niger, A. oryzae, A. wentii, Penicillium citirinum, P. frequentans, P. expansum, P. digitatum, Mucor mucedo and Streptomyces. A. Aspergillus versicolor is the major sterigmatocystin producer, while A. nidulans, and A. rugulosus have also been recognised as major. Mycotoxin producing strains.(1)

Yellowed Rice Fungi

They are of four types.

A) Luteoskyrin and cyclochlorotine producing fungi: Luteoskyrin and cyclochlorotine are hepatotoxic and hepatocarcinigenic mycotoxins obtained from cultures of Penicillium islandicum.

B) Citrinin producing fungi: Citrinin is a representative mycotoxin among the yellow rice toxins and was first detected from Penicillium citrinum. Tne following citrinin producing fungi known at present are P. citrinum, P. viridicatum, P. implicatum, P. canescens, P. notatum, P. expansum, A. niveus and A. flavips.(2)

It is interesting to note that the species P. viridicatum and P. palitans are producers of both citrinin and ochratoxin A.

C. Rugulosin producing Fungi: Rugulosin, an anthraquinoid fungal metabolite was first isolated from mycelia of Penicillium rugulosum. Other rugulosin producing fungi are P. brunneum and P. tardum.

D. Citreoviridin Producing Fungi: Citreoviridin is a neurotoxic mycotoxin produced by certain strain of Penicillium citreoviride. Other species which produce citreoviridin are P. ochrosalmoneum and P. pulvillorum.

Patulin and Penicillic Acid Producing Fungi

Patulin was isolated from both Penicillium and Aspergillus sp. P. patulum, P. expansum, P. lapidosum, P. griseofulvum, A. clavatus, A. giganteus, and A. terreus.

Penicillic acid was first isolated from Penicillium puberulum. Producibility of this compound has been demonstrated in the following fungal species namely P. puberulum, P. sotoniferum, P. palitans, Aspergillus ochraceus, A. sulphureus and A. ostianus.

Griseofulvin Producing Fungi

It was first isolated from Penicillium griseofulvum.

The other fungi reported to produce griseofulvin are _Penicillium patulum_, _P. viridi-cyclopinum_ and _P. albidum_.(3)

Ochratoxin A Producing Fungi

Ochratoxins are metabolites of _Aspergillus ochraceus_. The known ochratoxin producing fungi are _A. ostianus_, _A. melleus_, _A. sulphureus_, _Penicillium viridicatum_, _P. commune_ and _P. variabile_.(4)

Rubratoxin producing fungi

Rubratoxins are toxic metabolites produced by isolates of _Pencillium rubrum_. Rubratoxin B is produced by _Penicillium rubrum_ and _P. purpurogenum_.(5)

Trichothecene Toxin Producing Fungi

Tricothecene, the group of Sesquiterpenoids previously known as Seripenes, include a number of fungal metabolites produced in cultures of various species of the genera _Fusarium_, _Cephalosporium_, _Trichoderma_, _Stachybotrys_. According to structural variation at the C-8 position and Taxonamic situation of the toxin producing fungi have divided the Toxic trichothenes into two types A (T-2 toxin, neosolaniol, HT—Toxin Diacetoxy sciripenol) and Type B toxins (Fusarenon-X, Nivalenol).(6)

Zearalenone Producing Fungi

Zearalenone (F-2 toxin) is an estrogenic mycotoxin produced by several species of _Fusarium_. It was first isolated from cultures of _F. graminearun_ invading stored corn, _F. roseum_, _F. nivale_, _F. sporotrichioides_ are also known to produce this Toxin.(7)

2. Microflora of Various Food

It is well known that cereal grains, stored grains and processed food of both plant and animal origin harbour large number of fungi under certain natural conditions. These Fungi can be divided into (1) Field Fungi (2) Storage fungi (3) Advanced decay fungi. The principal genera of field fungi are _Alternaria_, _Fusarium_, _Helminthosporium_, and _Cladosporium_. The microflora of stored fungi include _Pencillium_, _Aspergillius_ and _Sporendonema_. These stored fungi are the dominant type of moulds associated with stored seeds.

Fungi that invade cereal grain can be categorised into three groups which have different moisture requirements. These include (a) Field fungi which invade grain in the field before harvest and include species of _Alternaria_, _Fusarium_, _Cladosporium_, and _Helminthosporium_. (b) Storage fungi, which predominate in the grain during post-harvest storage and consist of primarily _Aspergillus_ spp. and _Penicillium_ spp. (c) Advanced decay fungi such as

Fusarium and Chaetomium, which grow after considerable deterioration has occurred. (8)

Table No 1: Cereal Grains Invade Fungi

Group	Organism	Occurrence and moisture requirements
Field Fungi	Alternaria Fusarium Cladosporium Helminthosporium	Field 20 to 25% moisture
Storage Fungi	Aspergillus Penicillum	Storage 13 to 18% moisture
Advanced Decay Fungi	Fusarium Chaetomium	Storage 20 to 25% moisture

Among the chief rice field fungi are Helminthosporium, Fusarium, Alternaria, Epicoccum are predominant.(9) Chief fungi which invade wheat are Fusarium graminearum, Cladosporium sp. Aspergillus candidus, Penicillium chrysogenum and Botrytis cinerea.(10) Major field fungi that invade corn are Alternaria, Cladosporium, Helminthosporium, Trichoderma and Fusarium.(11) Similarly the fungi which invade sorghum are Fusarium, Cladosporium, Helmnthosporium, etc.

10

Meat represents ideal culture medium for many micro organisms due to its high moisture content and rich nutritive value. Cold meat allows the growth of psychrophilic fungi such as _Mucor recemosus_, _Rhizopus_ sp, _Pencillium expansum_, _Cladosporium herbarum_.

3. Source of Food Contamination

Mycotoxins are considered to be one of the most dangerous contaminants in food and feeds. Toxicogenic mould spores are almost universally present in the atmosphere in the soil and in agricultural crops and products. The main entry of mycotoxins into human and animal food chain is from agricultural products such as cereal grains, oil seeds and products derived from these sources. The three genera _Aspergillus_, _Pencillium_ and _Fusarium_ are considered to be the most significant toxigenic mould at the present time.(11)

4. Occurrence of Mycotoxin

Mycotoxin concentration in cereals is high enough to cause toxic symptoms on both humans and on domestic animals have been reported in years. There are some well documented cases of occurrence Fusarium Toxins in USRR (1942-47),(12) and Finnish grains and feeds during 1970. Ylimaki (1979) et al., (1979) analysed 230 samples from different parts of the Finland and showed that 24 of the 230 samples contain Zearalonone (ZEN) and four samples contains T-2 Toxin at

11

concentration ranging from 100 to 40000 u g/kg and 10 to 50 ug/kg respectively.

Later it was suspected that the mycotoxicosis in the Finland occured in 1982, 1983, and 1984. The summer and autumn of 1984 were rainy and generally wet in Finland creating favourable conditions for the growth of mould (14). Karppanen et al., (1985), analysed 167 samples of commercial feed and different species of cereals in 1984 crop. They had found that the cereals and feed samples were contaminated with Trichothecenes. The Trichothenes detected were Deoxyvinalenol (DON), nivalenol (NIV), T-2 and HT-2 Toxin.(14)

In many countries DON, NIV and ZEN produced by Fusarium, Graminerium are the major toxicants in cereals and feeds,(16) DON is the most important mycotoxin in grains in the U.S.A., Canada, Australia, S.Africa, Italy and U.K. ZEN is also widely distributed throughout the world in cereals and feeds.(14)

During June to September, 1987 an outbreak of a gastrointestinal disorder occurred in the Kashmir Valley especially in the city of Srinagar and the surrounding areas. The outbreak was identified as being due to Trichothecene mycotoxins present in bread made from rain damamged mouldy wheat. The 4 mycotoxins detected were Ergosterol, DON, NIV, T-2, Ac-DON.(17)

Enormous amount of information on natural contamination by mycotoxins have been collected mostly in the U.S.A. Important naturally occuring mycotoxins contaminated food stuffs are listed below.

Aflatoxins   : Peanuts, Rice, Wheat, Corn

Sterigmatocystin : Grain samples, moldly stored rice

Ochratoxin A   : Wheat, Barley, oat, corn, rice

Citrinin   : Barley, wheat, oat, corn, Rye

Patulin   : Apple juice

Penicillic acid   : corn

T-2 Toxin   : corn

Deoxynivalenol   : Barley, wheat

Zearalenone   : Corn, feed stuffs

5. Detection and Identification

Various methods have been used for detection and identification of the mycotoxins. They are: 1) physical methods; 2) Chemical methods; and 3) Immunoassay methods.

5.1 Physical Methods

Thin layer Chromatography has play an important role in the purification of many naturally occuring trichothecenes and has been used for identification in different samples.

5.2 Chemical Methods

High performance liquid Chromatography. The method used to analyze these compounds generally have low sensitivity and need extensive instrumentation. A good method should be very specific, sensitive and relatively simple to operate. One method which has gained great popularity for analysis of mycotoxins is high performance liquid chromatograph (HPLC). HPLC methods combined with fluorescence and UV detection system have shown good sensitivity and excellent reproducibility. Because of high sensitivity, sample must first be subjected to an extensive clean-up treatment before separation by HPLC.

5.3 Immunoassay Methods

Recent developments, for detection of mycatoxins are immunoassay which involves the interaction of Biological materials. Two assays systems have been developed i.e. radioimmunoassays (RIA) and Enzyme-linked immunoassay (ELISA).

5.3.1 Radio Immunoassay (RIA)

The RIA procedure involves simultaneous incubation of the unknown sample or known standard dissolved in phosphate buffer with a constant amount of labeled Toxin and a specific antibody.

Several methods have been used for the separation of free and bound Toxin. The ammonium sulphate precipitation method is simplest and has been used for T-2 Toxin. Analysis of AFB_1, has been achieved by either the double antibody technique or solid phase RIA method in which the IgG is conjugated to CNBr-activated sepharose gel. A dextron-coated charcoal column which has been commonly used for RIA for other compounds.(18)

5.3. 2 Enzyme-linked Immunoassay [ELISA]

In ELISA of mycotoxins, specific antibodies are first coated to a solid phase, such as a microplate or a polystryrene tube. Among several methods tested for coating antibody to the microplate, the Glutaraldehydes and bicarbonate methods appears to be most suitable for the assay. Once the antibody is dried on the plate, and is washed before use. The sample solution or standard Toxin generally incubated simultaneously with enzyme conjugate. The plate is washed again, and the residual enzyme bound to the plate or tube is then determined by incubation with a substrate solution containing H_2O_2 and appropriate oxidizable chromogens. The resulting colour is measured spectrophotometrically or by visual comparison with standards. The oxidizable chromogens used for ELISA of mycotoxins include 0-Toluidine or 5-amino-salicyclic acid.

In general ELISA is 10 to 50 times more sensitive

15

than RIA when purified mycotoxin are used. ELISA is more advantage when radioactive ligand of the Toxin is not available.(18)

Table No 2: Sensitivity of RIA for Mycotoxins.(18)

Mycotoxins	Standard range (ng)	Detection limits ($\mu g/kg$)
AFB_1	0.5-5.0	5.8
AFM_1	5-50	5
T-2	0.2-2.5	1.0
Zearalenone	0.25-10	5.0

AFB_1= Aflatoxin B[1] AFM_1= Aflatoxin M[1]

Table No 3: Sensitivity of ELISA for Mycotoxins (18).

Mycotoxins	Standard range (pg)	Detection limits
AFB_1	25-1,000	3
AFM_1	25-1,000	0.25
OA	25-500	1-2
T-2	25-200	2.5

OA. Ochratoxin A.

16

5.3.3. Indirect Enzyme Linked Immunoassay for T-2 Toxin in Biological Fluids

An Indirect enzyme-linked immunosorbent assay (ELISA) which can be detected 0.2 to 1 ng of T-2 Toxin per ml in urine, serum, and milk has been developed. T-2 Hemisuccinate was conjugated to polysine which was then coated to a microtiter plate and incubated with rabbit anti T-2 antibody and sample extract. The amount of anti-T-2 antibody bound to the plate was then determined by reaction with goat anti rabbit IgG-peroxidase complex and subsequent reaction with the substrate. Samples spiked with T-2 Toxin were subjected to simple clean up procedure by passing them through a reversed-phase Sep-pak catridge.(18) The recoveries of tritiated T-2 Toxin added to the urine, serum, and milk sample were between 71 to 90% after clean up step.

In indirect ELISA, much less antibody is required for each analysis, hence it is more economical to perform than the direct ELISA. Indirect ELISA is adqeuate for monitoring trace amounts of T-2 Toxin in Biological fluids. Direct ELISA has always the problem of Enzyme instability in the preparation of T-2 Toxin-enzyme conjugate and during storage, the preparation of T-2 HS-PLL is relatively simple and the conjugate is also very stable.(19)

6. Mycotoxin Biosynthesis

Mycotoxins are secondary metabolites produced by a variety of filamentous fungi. They are secondary sesqui-terpeniod metabolites of fungi. The trichothecene and aflatoxin are the most important members of this very diversed class of Toxins.

Mycotoxin are produced in the late exponential or early stationary phase of growth, Moisture and temperature are two factors that have a crucial effect on mould growth and colony production. Generally mould growth favoured by high humidities in combination with high temperature. Temperature is very important factor affecting mycotoxin formation. Fusarium species produce Zearalenone and the Tricholhecene (include T-2) Toxins are capable of producing at low temparature.

They can be mainly derived from the following pathways :-

1) Mycotoxins derived from amino acids

2) Mycotoxins derived from aromatic pathway

3) Mycotoxins derived from Acetate Mevalonate pathway

18 .

6.1 Mycotoxins Derived from Amino Acids

In the pathways of amino acid metabolism in higher plants, decarboxylation of amino acids commonly occurs to form corresponding amines. These amines are regarded as 'proto-alkaloids' and they act as precursors for alkaloid biosynthesis. However decarboxylation is less important in fungi and various amides or peptides are produced in fungi in place of the amines or alkaloids formed in higher plants. Some important mycotoxins containing nitrogen are known to be produced from Amino acids.

Sporidesmin from *Pithomyces chartarum* is one such nitrogenous metabolite which also contains sulfur in its structure. Obviously all the sporidesmins are basically derived from two amino acids, Tryptophan and alanine forming cyclodipeptide.

Gliotoxin, an antibiotic isolated from *Myrothecium Verrucaria* (*Gliocladium fimbriatum*) also contains thiadioxo-piperazine moiety. It was shown that the phenylalanine and serine and also a C_1 unit from methionine provide the carbon skeleton of Gliotoxin.(20)

Aspergillic acid and neoaspergillic acid (produced by *Aspergillus flavus* and *A. oryzae*) or pulcherriminic acid produced by *Candida pulcherrina* are formed from dioxopiperazine.

19

Roquefortine, a 2,2,5 Dioxopiperazine compound formed from Tryptophan and histidine was isolated from _Penicillium roqueforti_ has tremogenic acitity.(21)

Ergot alkoloids contain peptides linked to carboxyl group of lysergic acid. These peptides are of derived from amino acids. Lysergic acid and isolysergic acids are known to be derived from Tryptophan by condensation with one "isoprene" unit formed from mevalonic acid.

6.2 Mycotoxins Derived from the Aromatic Biosynthetic Pathway (Shikimic acid pathway).

An important role of the Shikimic acid pathway in secondary metabolism is to provide intermediates for the biosynthesis of aromatic compounds including amino acids. The shikimic acid pathway plays a relatively small part in secondary metablism in fungi.

Xanthocillin has been isolated from _Penicillium notatum_ and also from _Aspergillus chevalieri_. It exhibits heptotoxicity in experimental animals. This compound is an unusual isocyanide and is usually obtained as a mixture of xanthocillin X and Y.

6.3 Mycotoxins Derived from the Acetate Mevalonate Pathway

Acetate melonate pathway for the biosynthesis of various compounds including phenolic compouds. The most important mycotoxins derived from this pathway are citrinin Trichothecenes, Aflotoxins.

Citrinin is an yellow crystalline antibiotic compound isolated from _Penicillium citrinum_ and its has P-quinone methide structure.(22) Ochratoxin A is a toxic metabolite of _Aspergillus ochraceus_. Ochratoxin A is a dihydroisocoumarin carboxylic acid linked through its carboxyl group to the amino nitrogen of phenylalanine.(23)

Among the fungal Heptaketides, an antifungal antibiotic Griseofulvin, was isolated from _Pencillium griseofulvum_ and subsequently isolated from a large number of penicillium species. Griseofulvin was derived from acetate.(24) Aflotoxin biosynthesis can occur through the acetate-malonate pathway, eventhough this mycotoxin contain coumarin skeletion. As general rule, coumarin compounds are formed through the aromatic pathway of biosynthesis involving phenylalanine, cinnamic acid and shikimic acid as intermediates. Biosynthesis of aflatoxin in acetate-malonate pathway takes place via C_{20} anthraquinone.(25)

Jones and Lower (1960) were first showed that acetate $1-^{14}C$ and mevalonate $2-^{14}C$ were incorporated into the skeleton of trichothecolone. Incorporation of mevalonic acid and farmesol pyrophosphate doubly labelled with ^3H and ^{14}C into trichodermal, Trichothecins and Trichothecolone indicate that a 6-7 Transfarmesol pyrophosphate is a precursor of these compounds.(27)

The Biosynthesis of Trichothecenes proceeds from the

alicyclic hydrocarbon trichodiene which is a cyclization product of farnesyl pyrophosphate.(28)

7. Some Aspects of Structure and Activity Relationship in Mycotoxins

It has been established that compounds containing a lactone ring possess a wide range of cytotoxic and carcinogenic properties. Since a small membered lactones is subject to nucleophilic attack, the biological and toxicological activities of these lactones may be attributed to their alkalating ability.

In adition to lactones, epoxides and halo-esters, are important alkylating agents. It is known that Diepoxides are more frequently carcinogenic than monofunctional ones. It has been demonstrated that diepoxybutane reacts easily with Guanine at N-7 position in vitro. With many fungal metabolites, Trichothecene possesses an epoxy group. It is interesting that biologically active trichothecenes lose their activity when they lose their epoxy group.

Chlorinated mycotoxins such as ochratoxin A, griseofulvin and cyclochlorotine are highly hepatotoxic. Ochrotoxin B, a dechloro derivative of ochratoxn A, has no Toxicity.

8. Trichothecene Mycotoxins

Trichothecenes are secondary metabolites produced by

several groups of fungi including Fusarium, Trichothecium, and Myrothecium sp. Biosynthesis of trichothecene proceeds from the alicyclic hydrocarbon trichodiene, which is a cyclization product of Farnesyl pyrophosphate.(28) The 15 carbon skeletion of the trichothecene suggested that these compounds are susquiterpenoid.

All naturally occurring Trichothecenes mycotoxin contain an olefinic bond and an epoxy group at 9, 10 and 12, 13 respectively and may therefore be characterized as 12, 13 epoxy trichothecene. Inaddition all have at least one hydroxyl or ester group at position 4. Naturally occurring Trichothecenes are colourless, crystalline, optically active solids which are generally soluble in moderately polar organic solvents but only very slightly soluble in water.

Table 4 : Trichothecene Producing Fungi(29)

1)	F. Sporotrichioides F. equiseti	T-2, HT-2, neosolaniol, Diacetoxyscripenol
2)	F. graminarum F. Culmorum	Nivalenol Deoxynivalenol 3-Acetyldeoxynivalenol
3)	Cephalosporium Crotocigerum	Crotocin

Macrocyclic trichothecene such as Verrucarin A and roridin A are most toxic agents, while Trichothecin, trichodermin and crotocin are very weak in comparison with

other Toxions.

All trichothecenes possess skin irritant or skin
necrotizing activity for animals. The type-A Toxins (T-2
Toxin, HT-2 Toxin) are about ten times more toxic than the
type-B Toxins (nivalenol, fusaremon-X). The other important
characterstic symptoms are vomiting, Leucocytosis, and
Leucopenia in experimental animals. There is no marked
difference in lethal trichothecene doses are observed
according to different route of administration indicating
that both the orally and inter peritoneally administered
toxin are quite smoothly absorbable in animals.

9. T-2 TOXIN

Source: The most investigated trichothecene mycotoxin, T-2
Toxin is a secondary metabolite of fungi belongs to
different species of Fusarium and some species of
Trichothecium Cephalosporium and Stachybotrys.

9.1 Precursors of T-2 Toxin

The following precursors of T-2 Toxin are able to
coverted Toxin in F. sporotrichioides MB5493.(29)

1. 3 ∝ -Hydroxytrichothecene (isotrichodermol)

2. 3∝ -Acetoxytrichothecene (Isotrichodermin)

3. Tricho - 9 -ene 2∝ , 3∝ , 11∝ - triol

4. Tricho - 10 ene - 2∝ , 3∝ , 9∝ , Triol (Trichotriol)

5. Tricho- 10 ene - 2∝ , 3∝ , 9 β - Triol.

9. 2 Chemical Nature of T-2 Toxin

Molecular formula C_{22} $H_{34}O_9$ and F.W. 466.5 Structurally they are sesquiterpenes and have an epoxy ring at C-12,13. The toxin contained one free acetylable hydroxyl group and an definite bond which was easily hydrogenated to a dihydrocompound. T-2 toxin was shown to be - 3 -Hydroxy 4 -15-Diacetoxy 8 -C3-methyl butyl Hydroxy-12,13-epoxy 9- Trichothecens.Trichothcene contain olefinic double bonds between 9 and 10. Reducing the C-9, C-10 double bond results in a slight decrease in activity. They are relatively stable, optically active solid. The Toxin are extractable with Polar solvents but have low solubility.

T-2 Toxin posses a tetracylic 12, 13, epoxy - Trichothece - 9 enee core structures. This core structure is formed from Farnesylpyrophosphate. In a reaction catalysed by Trichodiene synthase, Farnesylphyrophosphate cyclizes to form Trichodiene, the parent hydrocarbon of the Trichothecene. Among remaining steps in the pathway of T-2 Toxin are six oxygenations which are catalyses by molecular oxygen dependant enzyme.(30)

It was reported that carbonyl oxygen of the acetate at C-4 & C-15 and of the isovalerate at C-8 were derived from water. The oxygen at position 1, the 12, 13 epoxide and the Hydroxyl group at C-3, C-4, C-8, C-15 were all
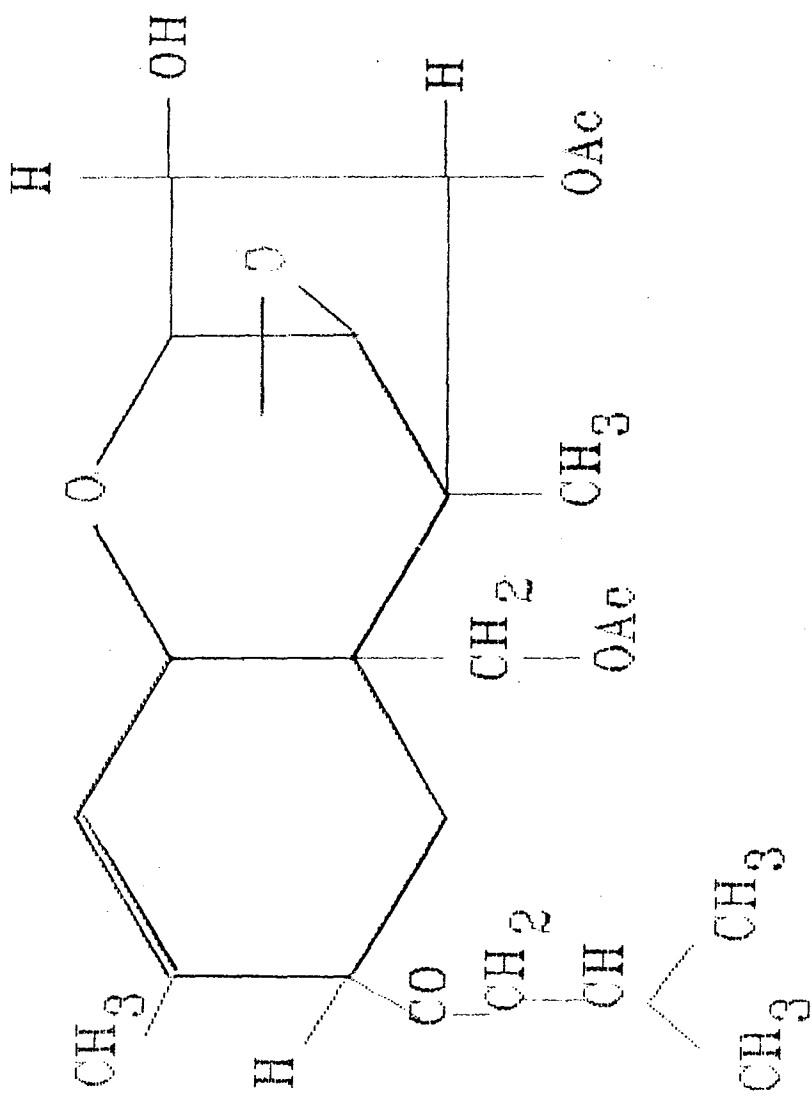
T-2 toxin

derived from molecular oxygen.(31) They all show atleast Hydroxyl or ester group at 4th position.

10. Regulation and Control

Formation of mycotoxins can be controlled to a certain extent by controlling the factors that affect formation of mycotoxins. Mycotoxin production is favoured by high humidity and high water activity. For eg. Aflotoxin production is favoured by temperature of $25^{\circ}C$ to $30^{\circ}C$.Penicillium sp. can produce putalin, penicillic acid and ochratoxin at temperature from 0 to $31^{\circ}C$ where A ochraceus does not produce ochratoxin or penicillic acid below $12^{\circ}C$. Fusarium sp. can produce Zearalonone and Trichothecenes at temperatures below $10^{\circ}C$ and near or even below freezing point. If moisture contents can be reduced and maintained at sufficiently low levels, mould growth and mycotoxin production will not occur, and controlling storage temperature may also be a means of preventing production of certain mycotoxins.

Mycotoxic moulds are highly aerobic organisms and these have a requirement for oxygen. A low oxygen concentration and high concentration of other gases may depress the mould growth and mycotoxin formation. Sanders (1968) et. al found that at constant temperature, high level of CO_2 and low relative humidity prevents aflatoxin production.

26

The presence of competing microorganisms can restrict fungal growth and mycotoxin production. Aflatoxin production by A. flavus is less when grown in mixed culture with A. niger than grown in pure culture. The other moulds such as A. oryzae and Rhizopus nigricans have also been reported to inhibit A. parasiticus and aflatoxin production. A number of lactic acid Bacteria can also grow competitively with A. parasiticus and resulted in less aflatoxin production.

Mold growth can also be prevented through the use of antifungal and Antimicrobial agents. A number of chemicals that have anitmycotic properties are approved for use in foods, several of those substances are organic acids or their salts, and include: Sorbic acid and sorbate, propionic acid and propionate, benzoic acid and benzoate and acetic acid and its derivatives. In adition, the phenolic antioxidants particularly BHA have antimycotic properties. Plant products like Cinnamon, cinnamon oil, clove and clove oil have been shown to be strong antimycotic properties.(32)

'Superactive' charcoal was assessed for efficacy in decreasing the lethality of both oral and parenteral exposure to T-2 Toxin. Activated charcoal might be effective in decreasing lethality by binding Toxin metabolites. Activated charcoal has been used for the treatment of a wide variety of intoxication. Aflatoxicoses in goat and chicken has been successfully treated with activated charcoal.(34)

27

To control the mycotoxin contamination of food and feeds, the following four kinds of measures are basically needed.

1) prevention of the initial growth of moulds and subsequent contamination by mycotoxins.

2) Detection of mycotoxins in food materials and selective removal of the contaminated portions.

3) Inactivation or destruction of all Toxins either by chemical, physical and or enzymatic means.

4) Advance identification of possible new mycotoxin threats.(35)

10. 1 Research Activities Related to Mycotoxin Control

1) Determination of the agricultural practices which must be controlled to prevent contamination.

2) Search for mold-resistant plant varieties.

3) Development of plans for sampling commodities for contamination.

4) Development of new analytical methods and improvement of existing methods.

5) Development of reliable rapid screening methods.

6) Establishment of techniques for removing contaminated portions from a lot of food.

7) Development of procedures for ,detoxification of contaminated products.

28

8) Determination of the relationship between levels of contamination in animal feed and level in edible tissue, milk, or eggs.

9) Determination of the toxicological effects of mycotoxins in farm animals.

10) Establishment of the chemical and physical characteristic of Toxic fungal metabolites which are potential food contaminents and which are as yet still of unkown chemical structure.

International interest in the Economic and public health problems associated with mycotoxin contamination of food and feeds have grown considerable. The Food and Agricultural organization (FAO) of the United Nations. The World Health Organization (WHO), and United Nations. Environment programme (UNEP) are undertaking review of the entire problem.(36)

11. Use and Applications

The very considerable investment on the part of several large pharmaceutical firms in trichothecene research was apparently prompted by the hope of finding an effective antitumor agent. The antifungal activity has been exploited to a moderate degree. In particular trichodermin has been recommended for treatment of Candida albicans infection in human beings. It has also been claimed to control Fusarium rot of potatoes and to be active against several other plant pathogenic fungi. Trichodermin has been applied to cotton seeds and plants for prevention of wilt.

T-2 Toxin reportedly have been used as a biological warfare agent in South East Asia and Iran, and human exposure to T-2 Toxin as a result of Biological warfare is evidenced by the detection of these Toxins in samples collected in war zones.

12. T-2 TOXIN AND BIOLOGICAL RESPONSE

12.1 General Toxicity

T-2 Toxin, the trichothecene mycotoxin is believed to be the causative agents of number of mycotoxicoses affecting both human and domestic animals. The most common symptoms are food refusal, vomiting, skin inflammation, nervous disorders, leukopenia, thrombocytopenia, haemorrhage etc. The toxic effect of the mycotoxin is characterized by an injury to haemotopoietic and immune competent systems, and disturbed functions of the cardiovascular system, and gastro-intestinal-track.(37) Acute intoxication of T-2 Toxin in Rats observed with decrease in serum free fatty acid levels and increased Blood urea nitrogen levels (BUN)and increased activity of Lactate dehydrogenase (LDH), Alanine aminotransferase (ALT), Aspertate aminotransferase (AST) and creatinkinase (CK) levels(38). In vitro studies has shown that T-2 Toxin disturb the electron-Transport system in mitochondria(39). Administration of T-2 Toxin results in increase of liver weight significantly. [3][H] Thymidine incorporation into liver DNA was not significantly alter in animals treated with T-2 Toxin, while RNA levels in Rat liver was significantly increased after T-2 Toxin treatment.(40). LD_{50} value of T-2 is 3.8 mg/kg in Rat and 3.0 mg/kg^{-1} in guinea pigs.(41)

LD$_{50}$ Values (mg/kg) of T-2 Toxin(42)

Mouse	I.P	5.2
Swine	I.V	1.21
Chick	P.O	4.0
Mice		10.5

12.2 The Haematopoietic System with T-2 Toxin

A concurrent decrease in haematocrit values was observed in the treated animals which may indicate that the decline in serum protein was caused by blood volume changes rather than a generalized inhibition of protein synthesis. T-2 Toxin causes damage to haematopoitic tissue such as bone marrow and spleen, it is possible that the decline in haematocrit value is the result of the suppressive effect of T-2 Toxin on haematopoietic tissue.(38)

T-2 Toxin is liophilic in nature. It apparently enter cells easily, and in platelets, it may rapidly alter surface related functions, of these the most pertinent to the clinical picture of intoxication is the aggregation abnormality. The haemorrhagic diatheses although said to be due to damaged blood vessels, may be caused by direct, rapid and dose related effects of T-2 Toxin on platelet function. (43)

T-2 Toxin is able to inhibit the aggregation of

bovine platelets suspended in homologous plasma following stimulation either with ADP or collagen. Inhibitory action of the mycotoxin depends not on the length of the pre-incubation periods of the platelets with the Trichothecene but on the amount of Toxin to which the platelets are exposed. In addition to the release of contents from the platelet granules, mycotoxin can also cause a significant decrease in the circulation levels of specific coagulating proteins. When both platelets and coagulation protein are functionally impaired, it would possibly be unable to produce an adequate haemostatic responses to trauma.(44)

It was suggested that free radical mechanism is involved in the haemolysis caused by H_2O_2 and polyoxyethylene surfactant. The similarity of time dependence curve of haemolysis induced by T-2 toxin to the curve observed for the haemolysis by free radical mechanism suggested that the same mechanism is responsible for the haemolysis induced by T-2 toxin. This suggestion was substantiated by the fact that histidine and methanol which are known to interfere with free-radical reactions, attenuated haemolysis caused by T-2 toxin. The inhibition of haemolysis by mannitol, specific OH quencher suggests that OH radicals may be involved in the haemolysis process.(45)

12.3 Enzymatic Changes with T-2 Toxin

T-2 toxin has ability to interact with thiol groups

33

of enzymes (46). Single oral administration of T-2 toxin at the LD $_{50}$ (3.8 mg/kg body wt), to Wistar rats inhibits the activity of majority of lysosomal enzymes, mitochondrial succinic dehydrogenase, and moderately decrease the UDP-glucuronsyltransferase and glutathione-transferase(47).

Administration of T-2 toxin significanty increased lipid peroxidation in rat liver as measured by formation of malondialdehyde. The malondialdehyde content increased significantly as early as 8 hr. after an acute single dose of T-2 Toxin. Rather multiple adminstration of moderate doses of T-2 Toxin in these rats enhanced lipid peroxides significantly.

T-2 Toxin is known to bind SH-proteins. The epoxide group of T-2 Toxin competes with the substrates of glutathione- s - transferase. These may result in decreased content of GSH. Prevention of lipid peroxidation is primarly connected with GSH metabolism. It is possible that T-2 Toxin reduces the level of GSH which is required for the elimination of peroxide radicals. The activity of all the three glutathione-shuttle enzymes (Glutathione Peroxidase, glutathione reductase, and Glucose-G-phosphate dehydrogenase) which markedly increase in all the stages of T-2 Toxin treated rats in short term experiments.

Glutathione peroxidase and catalase the decomposition of the highly reactive peroxidase radicals to their

corresponding alcohol with reducing equivalents of GSH. Glutathione reductase in turn restore the availability of GSH with reducing equivalents of NADPH through pentose-phosphate shunt pathway. A significant increase in GSH-shuttle enzymes in T-2 treated animals appears that T-2 Toxin may generate reactive oxygen radicals. Such generation of free radicals is responsible for haemolysis of erythrocytes induced by T-2 Toxin in vitro. Generation of free radicals by T-2 toxin could induce lipid peroxidation. Induction of lipid peroxidation may be one of the possible causes of hepatotoxicity of T-2 toxin. The activity of Glutathione-s-transferase in liver homogenate decreases in T-2 toxin treated rats. It is possible that hepatotoxicity of T-2 Toxin may be due to depletion of GSH and induction of lipid peroxidation in liver. (48)

T-2 toxin and other trichothecenes may be regarded as potential non-competitive inhibitors of lysosomal enzymes. The damage caused by T-2 Toxin to the surface of endoplasmic reticulum membrane may be manifested in the inhibition of mono-oxygenase system activity and simultaneous increase in the activity of UDP-glucuronosyltransferase and epoxide hydrolase as a result of their conformation state, increase in the membrane permeability. It has been demonstrated that induction of conjugated enzymes, completely or partially, prevents acute toxicity of T-2 Toxin while inhibition of conjugated enzymes is responsible for higher toxicity of T-2

Toxin.(48)

Subacute Toxicity of T-2 Toxin results in sharp reduction in the level of cytochrome P-450, a key component in the system of microsomal oxidation. Furthermore, there is reduction in the microsomal protein level and in rate of aniline hydroxylation and also decrease occurs in the activity of carboxyesterase involved in deacetylation of trichothecene mycotoxin in Rat liver.(49) In contrast activity of epoxide hydrolase, an enzyme thought to be primarily responsible for detoxification of epoxide containing compounds increased considerably.(48)

The study of activity of enzymes connected with different cellular structure and representing three main enzymatic lines of cell protection-microsomal oxidation, conjugation and cytosolic enzymes with high hydrolytic potential, demonstrated a subacute toxic effect of T-2 Toxin in producing a significant inhibition of the majority of these enzymes(48).

12.4 T-2 Toxin Metabolism and its Detoxification

T-2 Toxin was readily metabolised in rat liver homogenates and intestinal tract, oral administration of T-2 Toxin was found to be excreted in rats as HT-2 Toxin, neosolaniol, and several other unknown metabolites.(50) The activity of T-2 Toxin hydrolysis was highest in the microsomes of liver. It is highly possible that C-4

deacetylation of T-2 Toxin is a major metabolic pathway in the liver. C-4 acetyl group is more susceptible to hydrolysis than other acetyl substituents. Deacetylation at C-4, results in stepwise conversion to T-2 tetrol via 4-deacetylneosolaniol(50). T-2 Toxin was hydrolysed selectively to HT-2 Toxin by the microsomal carboxyesterase. The C-4-acetyl residue of T-2 Toxin can be selectively removed by enzymes located in microsomes and from HT-2 which was slightly toxic than parent. The T-2 was biotransformed to HT-2 Toxin by the liver S-9 fraction(51)

The substrate specificity of microsomal nonspecific carboxylase (EC 3.1.1.1) showed that C-4 acetyl residuces of T-2 Toxin was selectively hydrolyzed by microsomal esterase to yield the C-4 deacetylated metabolite HT-2 Toxin. As far as the structure-activity relationship of hepatic carboxylase is concerned it appears that the enzyme attacks the C-4 acetyl residue of Trichothecenes, and that the substitutients at C-3 and C-8 play in important role in the enzymatic hydrolysis of the C-4 acetyl residue. (49)

13. Some Immunological Aspects with T-2 Toxin

T-2 Toxin is well known inhibitor of both DNA and protein synthesis and there by damaging proliferating tissue of the haemopoietic system. Treatment with T-2 Toxin causes significant transistory decrease in thymus and spleen weight, and a decrease in the number of bone marrow cells, lymphocytes, leucocytes and neutrophils. The damage caused by T-2 toxin to the cells of bone marrow, spleen, thymus and lymph nodes, leads one to suspect the functional abnormality of the immune system.(52) The reduction in the number of lymphocytes may be the primary cause of impaired immunity. Lafarge-Frayssinet_et al., 1979 showed that responsiveness of spleen and thymus cells to mitogen is severely impaired in the presence of T-2 Toxin. Gastric administration of T-2 Toxin results in an impairment in neutrophilic function (54).

T-2 Toxin alters several leucocytic functions both in vivo and in vitro. T-2 toxin inhibits chemotaxis, phagocytosis, and the generation of chemiluminescence in granulocytes. It is well established that all the above mentioned leukocytic functions depend on the intactness of the membrane. The toxin might intercalate into phospholipid to either block the receptors or to modify the surface glycoprotein (55). It has also been shown that T-2 toxin inhibited phogocytosis and chemotaxis in polymorphonucellar

cell.   Toxin acts directly without being metabolised. (56)

The lack of effect of T-2 Toxin in hepatic tissue could be explained by the fact that T-2 Toxin as well as other epoxides are reactive towards SH groups. Liver cytosol has glutathione Transferase which is able to catalyse the conjugation of Trichothecene with GSH.(57)   The amphipathic behavior of T-2 Toxin suggested that cellular hydrophobic sites such as membranes are first possible point of interaction.

T-2 Toxin treatment increases the metabolic capacity of mouse peritoneal cells and also increases the macrophage cells in murine spleen.(49)   The in vitro administration of T-2 Toxin, has stimulatory effect on various parameters of macrophage metabolism.   The intracellular levels of both glucuronidase and lactate dehydrogenase were significantly enhanced.   The high levels of these hydrolytic enzymes and increased oxidative metabolism are characterstic of inflammatory macrophages.   It was also shown that an increase in phagocytic capacity of murine macrophages.(58)

13.1Humoral Response and Antibody Synthesis with T-2 Toxin

T-2 toxin has been associated with enhancement of certain components of the immune system.   Administration of T-2 Toxin enhanced antibody synthesis against antigen such as polyvinyl pyrrolidone or DNP-ficoll (Rosensten 1981) (59).   But it depressed anti-SRBC antibody formation and

prolonged allograft survival. These results show that during antibody formation, T-2 Toxin interferes differentially with the response to different antigens.(59)

The administration of T-2 Toxin to animals resulted in the decline of IgM and IgA antibodies and also suppressed IgE and IgG formation.(60)

13.2 Skin Response with T-2 Toxin

T-2 Toxin is known as a potent skin irritant. It causes lesion of skin ranging from slight redding to necrosis. The threshold for irritance (erythrema) and oedema was approximate $0.1 \mu g$ in all species. It has been reported that skin of rabbit and guinea pig were more sensitive to the effect of T-2 toxin than rat skin.(61) Topical application of T-2 Toxin was the most efficient in producing skin lesions. Topical application of T-2 toxin results in necrosis in all lymphoid, haemopoitic tissue and germinal region of intestinal crop.

13.3 Lymphocyte Response and Macromolecular Synthesis with T-2 Toxin

T-2 Toxin acts directly without being metabolised in lymphocytes.(53) T-2 Toxin is taken up by lymphocytes in a saturable manner. The amphipathic behaviour of T-2 toxin suggests that cellular hydrophobic sites such as the membranes are the first possible points of interaction.(62)

In dividing lymphocytes the earliest demonstratable T-2 Toxin effect could be measured is decrease in total protein synthesis. The effect on RNA synthesis takes longer time to demonstrate, while effect on DNA synthesis takes atleast one cell cycle for evidence. The decrease in nucleotide incorporation by T-2 Toxin treated cells may also be interpreted as inhibition of nucleoside transport. However T-2 Toxin can inhibit DNA synthesis at a much lower concentration than that can inhibit thymidine uptake. The higher the T-2 toxin dose, the faster the effect. The lower the dose, the longer the effect takes to be detectable.(63)

T-2 toxin inhibits DNA and protein synthesis both _in vivo_ and _in vitro_. T-2 toxin inhibit DNA synthesis measured as ^{3}(H)Thymidine incorporation and transformation to lymphoblast on PHA stimulated HPBL (Human Pheripheral Blood Lymphocytes). The inhibition of DNA synthesis in HPBL by the T-2 is dose dependent.

Several authors demonstrated that T-2 Toxin and other trichothecene inhibit protein synthesis by blocking peptidyl transferase (64) or by destroying polysomes.(65) These effects on macromolecular synthesis are possibly responsible for the impairment of the immune system. Target organelle of T-2 Toxin is the 60s ribosomes subunit of eucaryotes and good correlation was observed between inhibition of protein synthesis and affinity to ribosomes. It was also well

41

established that trichothecene Toxin inhibited protein synthesis and did so by binding to Eucaryotic ribosomes. It has been shown that inhibition of protein synthesis by binding to 60s subunit of the ribosomes is the primary mechanism of T-2 cytotoxicity.(65)

When the cells were incubated with [3][H] T-2 a significant increase in the quantity of T-2 associated with the cells occurred during the first 30 min, this increased further from 10-16 hrs and decreased after 24 hrs. The longer the contact period, the greater was the accumulation of the Toxin within the cells.(66)

13.4 Effect of T-2 Toxin on lymphocyte Proliferation

T-2 Toxin acts on both mitogen stimulated and non-stimulated lymphocytes.(61) In rapidly dividing lymphocytes, T-2 has the early demonstrable effects. Several lymphocyte functions such as lymphokine production antibody response to T-dependent antigens, graft rejection have been shown to severely impaired after in vivo administration of T-2 toxin (Rosenstain et al., (1979). Full receptor occupancy is the first step towards expression of T-2 Toxin activity. This suggested that the cell membrane is involved in or modulate T-2 Toxin toxicity.(63) The extreme sensitivity of lymphocyte tissue to T-2 Toxin could be explained by the fact that these cells posses only low level of detoxifying enzymes such as glutathion

42

transferase or there is receptor for this toxin on the surface.(68)

Trichothecene have a broad spectrum of biological effects. They have been shown to be potent inhibitors of protein synthesis and they are considered as immuno-suppressor agents.

MATERIALS AND METHODS

I

T-2 Toxin with Molecular weight 466.5 was obtained from Sigma Chemicals. With this a stock solution containing 1 mg/ml was prepared using 6% ethanol.

Injection solution of T-2 toxin was prepared from stock solution diluting it with Phospate buffer saline. The injection solution was given intradermally to Wistar rats. Single doses of T-2 toxin were injected in the ratio of 100 ng and 100 pg to 200 grams body weight of experimental animals respectively.

Toxin conversant animals were sacrifised by cervical dislocation after 7 days, 14 days, and 21 days.

II - A

1. Glassware

All the glass-ware which were used in this present investigation were washed and sterelized using standard tissue culture techniques.

2. Culture Media & Supplements

RPMI - 1640 culture medium with L-Glutamine (himedia) is supplemented with

a. 2 mg% sodium bicarbonate

b. Penicillin 100 u/ml

c. Streptomycin 100 μg/ml

d. Gentamycin 50 μg/ml

e. Foetal calf serum was supplimented (10%) at the beginning of each experiment

3. T-2 Toxin stock solution was diluted serially in PBS to obtain 100 ng/ml and 100 pg/ml before adding to the culture.

4. Equipments and other facilities used

a. Surgical instruments

b. Centrifuge (Remi)

c. Membrane filters (0.45 μ pore size) (GF/C)

d. Micropipettes (Sigma, U.S.A)

e. Neubaur Haemocytometer (GDR)

f. Simple microscope

g. Liquid Scintillation sp L 81 801 (Backman)

h. Laminar flow Bench (Adair, Dutt & Co. India)

i. Filteration assembly

5. Miscellaneous assay reagents

a. Con-A (Sigma, USA)

b. Heparin - phenol free (Biochemical division, P. chest Institute, Delhi)

c. Sodium Bicarbonate (BDH)

d. Tryphan blue

f. Trichloro acetic acid

6. Scintillation fluid: Toluene based fluid 4g of 2-5-Diphenyloxazole (PPO) and 0.1 g of 1, 4 bis 2 (5

phenyloxazolyl) benzen/litre of toluene.

7. Radioactive materials: Stock solutions were prepared in PBS.

^{3}(H)-Thymidine sp. activity 6500 mci/mole. The radioactive compound was obtained from BARC, Bombay.

II - B

1.Experimental animals

Rats (Wistar strain) weighing 200 ± 10 gms were obtained from animal house, J.N.U.

2. The rats were seperated into two groups. One group remained as such without any treatment and this served as control. The second group was divided into three sub-groups. Each sub-group having a number of 6 rats, was administered with two different concentrations of T-2 Toxin. The animals were sacrificed after a single dose of T-2 Toxin administration at different time intervals Viz 7 days, 14 days & 21 days.

Lymphocytes from the spleens were prepared from both control and T-2 Toxin administered animals. The experimental protocol followed for the evaluation of T-2 toxin effects can be seen below. The spleen lymphocytes of control animals were assessed _in vitro_ for the effects of T-2 toxin. Whereas the spleen lymphocytes from T-2

administred animals served for both in vivo & in vitro evaluation of T-2 Toxin effect.

In second group of animals after, administration, for in vitro study the isolated lymphocytes were challenged with the Toxin in the culture medium. The similar doses were administered to animals for the assessment of Toxic effect in vivo. The splenic lymphocytes responses after the toxin administration served as in vivo system.

3. Preparation of the Spleen Cell Suspension (69)

Lymphocytes were isolated from 3-4 months old rats. They were sacrificed by cervical dislocation and their abdominal skins were swabbed with 70% ethanol. A small dorsiventral incision through the skin of the left flank was made. The skin was pulled apart exposing the spleen which was easily removable by making a small incision through Peritoneum. Some fatty or connective tissues attached to the organ were easily excised. The organ was amputated and kept in petridish containing 5 to 20 ml of RPM 1640 medium.

4. Handling of Lymphocyte in vitro

4.1 Preparation of Lymphocyte Suspension

A single cell suspension is obtained by cutting spleen into small fragments with scissors and then pressing it through a fine stainless wire mesh in RPMI 1640 medium.

47

The single cell suspension was transfered to a test tube with the aid of pasteur pipette and centrifuged at about 1400 to 1700 RPM. The residue remained in the centrifuge tube washed with lysis buffer and centrufuged till the white pellet was obtained and finally the cell suspension was made with a few ml of culture medium.

4.2   Preparation of Lymphocyte Cultures (Waithe & Hirshhorm, 1973)

Using Tryphan blue ex-clusion principle, viability of lymphocytes were checked and were counted in an improved Neubauer hemocytometer. Then, the cell population was adjusted to 2 x 10^6 cell/ml using more fresh medium supplemented with foetal calf serum (10%). Lymphocytes in the triplicate culture were incubated at 37°C in round bottom culture tubes containing 2 x 10^6 cell/ml in RPMI medium with/ without con-A and with/without T-2 Toxin. The optimal concentrations of Con-A was 20 μg/ml of culture were added.

4.3 Labelling of Culture DNA synthesis

The rate of DNA synthesis was determined by measuring the incorporation of ^{3}(H) Thymidine into TCA precipitate. Each culture was labelled by adding 1 μci of ^{3}(H) Thymidine in 10 μl of RPMI 1640 during 0, 30, 50, 60, 70 hrs.

5. Termination Labelling and measurement of Radioactivity

5.1 Termination of cultures - Cultures were terminated by
keeping them in the ice bath.

5.2 Isolation of labelled DNA (Waithe & Hirschhorn 1973)

a. 2 ml of cold TCA was added to the culture tube and
allowed to stand over night at $4^{\circ}c$.

b. Precipitate formed was suspended by vigorous agitation
and collected on a $(0.45\mu$ pore size) millipore filter
by carefully pouring the sample into the funnel of a
millipore assembly . Culture tubes were rinsed well with
TCA, adding these ringes to the funnel.

c. Suction was applied and the precipitate was rinsed with
additional 5 ml of TCA.

d. Filters were placed in scintillation vials, dried
completely after which 8-10 ml of Toluene based
scintillation fluid was added.

5.3 Counting

All the vials were uniformly counted in L 81 801
scintillation counter for 1 minute per vial.

6. Statistical Analysis

The results presented in tables are the means of
Triplicate cultures. Calculation done according to the
formula suggested by Koosis (1972).

RESULTS

1. Biosynthesis of DNA in lymphocyte culture stimulated with Con-A and without Con-A.

2. Optimal dose of mitogen Con-A for lymphocyte proliferation

3. Inhibition of DNA synthesis by T-2 toxin.

1. Biosynthesis of DNA in Lymphocyte Culture Stimulated with Con-A and without Con-A

To investigate the DNA synthesis in lymphocyte with Con-A and without Con-A, isolated lymphocytes were incubated in vitro with (20 μg/ml) of Con-A and without Con-A for 0, 30, 50, 60, and 70 hours and radio active precursors were added at respective time intervals. The incorporation of ^{3}[H] Thymidine into Con-A stimulated and non-stimulated lymphocyte was determined. The results were shown in Table I.

I. Con-A stimulated lymphocytes showed a maximum ^{3}[H] Thymidine incorporation into DNA between 50-60 hours. Con-A non stimulated lymphocyte showed maximum incorporation at 20-30 hours and 50- 60 hours. The incorporation of ^{3}[H] Thymidine into DNA of Con-A Stimulated lymphocyte was many fold higher than non-stimulated lymphocyte.

II. Con-A stimulated lymphocytes showed maximum incorporation at 50-60 hours. This is due to 'S' phase of cell cycle and maximum incorporation takes place only at 50-

60 hours indicated that all cells are synchronized.

In earlier investigations from this laboratory have shown that the duration of lymphocyte cell cycle, and investigations have been undertaken to find out the optimal dose of Con-A for higher proliferate index (Khannan,1979).

2. Optimal Dose of Mitogen Con-A for Lymphocyte Proliferation

Con-A at sub-optimal dose sharply varies in their stimulating effects. Optimal dose based on the study, (Bohidar,1988) a threshold value of 20 μg/culture concentration of Con-A was employed uniformly in all experiments.

3. Inhibition of DNA Synthesis by T-2 Toxin

To evaluate the effect of T-2 Toxin, experiments were designed to analyse the effect of T-2 Toxin in the DNA synthesis on the Con-A stimulated lymphocyte cultures.

An attempt was made to study the effect of T-2 toxin on proliferative lymphocyte in culture. Further experiments were designed to investigate the alteration, if any in DNA synthesis at 7 day, 14 day and 21 days treated animals with two different concentration of T-2 toxin (100ng, and 100pg/ ml of culture).

51

Table 1: Effects of T-2 Toxin with sitogen (Con-A) Stisulated Lysphocyte
and Non-Stimulated Lymphocyte in vitro
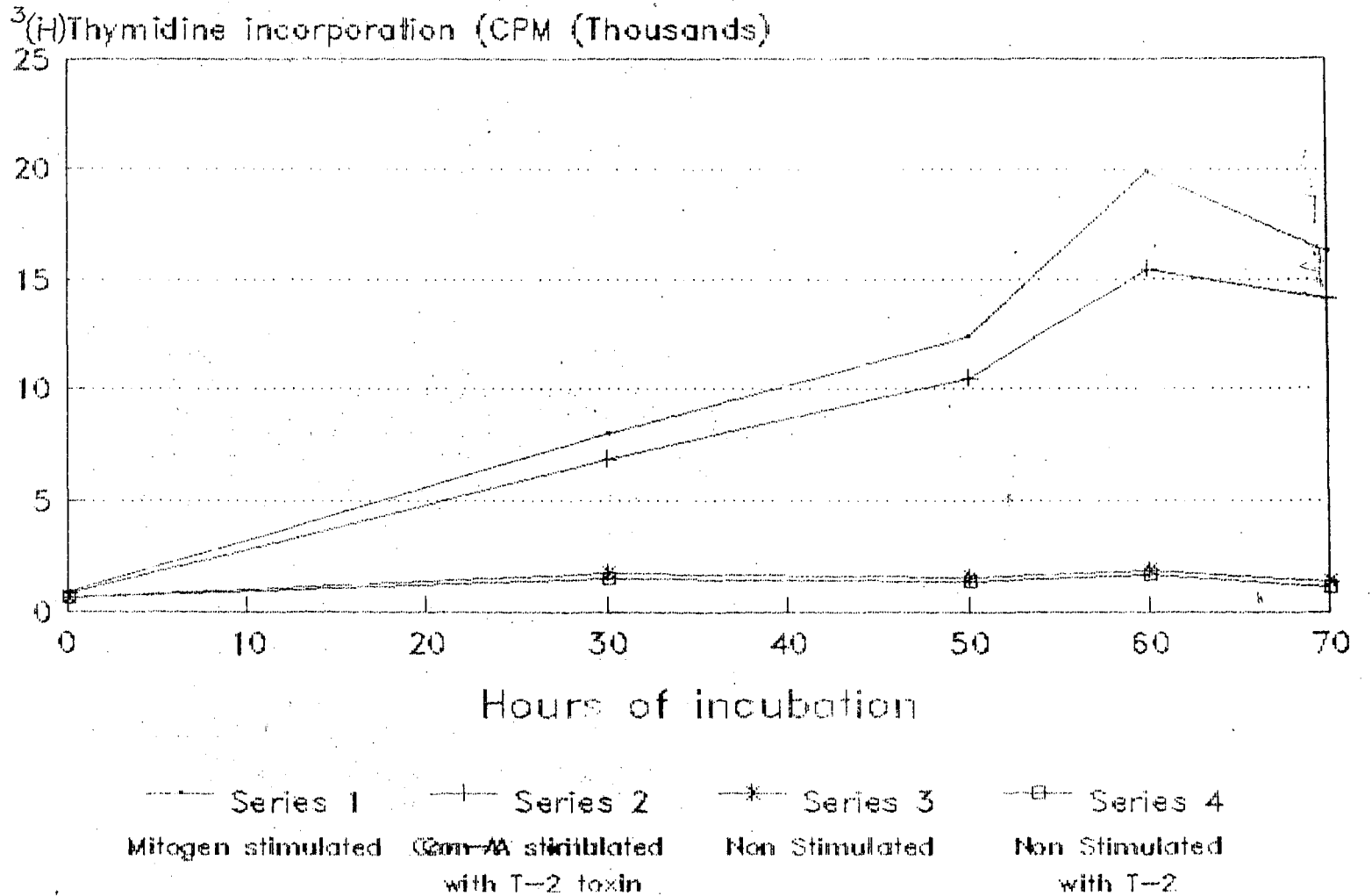
	^{3}(H) Thysidine incorporation (CPM ± S.D)									
Hours of Incubation	Mitogen Stimulated Lymphocytes (Con-A 20 μg/ml)	100 pg/ml	% of Change	100 ng/ml	% of Change	Non-Stimulated Lymphocyte	100 pg/ml	% of Change	100 ng/ml	% of Change
0h	951.25 ± 22	794 ± 49	6.72	674.33 ± 34	20.78	624 ±41	584 ±39	6.41	586 ±53	6.08
30h	7970 ± 49	6834 ± 49	14.25	3823.33 ± 43	52.02	1750 ±50	1465 ±53	16.28	812 ±40	53.6
50h	12364.75 ± 10	10463 ± 30	15.38	5457.33 ± 5	55.86	1526 ±34	1382 ±40	9.43	682 ±25	55.30
60h	19876 ± 17	15456 ± 44	22.23	8326 ± 19	58.11	1892 ±41	1696 ±22	10.35	654 ±70	65.43
70h	16246.25 ± 30	14128 ± 41	13.03	7871 ± 5	51.55	1368 ±52	1124 ±22	17.83	580 ±25	57.60

Values represent the sean, ± S.D. of four observations.

The same results are expressed in fig 1a &b

Effect of T-2 Toxin (100 pg/ml) on Con-A Stimulated Lymphocyte in vitro

^{3}(H)Thymidine incorporation (CPM (Thousands)

Hours of incubation

	Series 1		Series 2		Series 3		Series 4
	Mitogen stimulated		Con-A stimulated with T-2 toxin		Non Stimulated		Non Stimulated with T-2

Effect of T-2 Toxin (100 ng/ml) on Con-A
Stimulated Lymphocyte in vitro

Fig. 1B

^{3}(H)Thymidine incorporation (CPM)(Thousands)

Hours of incubation

─•─ Series 1	─+─ Series 2	─*─ Series 3	─□─ Series 4
Mitogen stimulated	Con-A with	Non Stimulated	Non Stimulated
	with T-2 toxin		with T-2
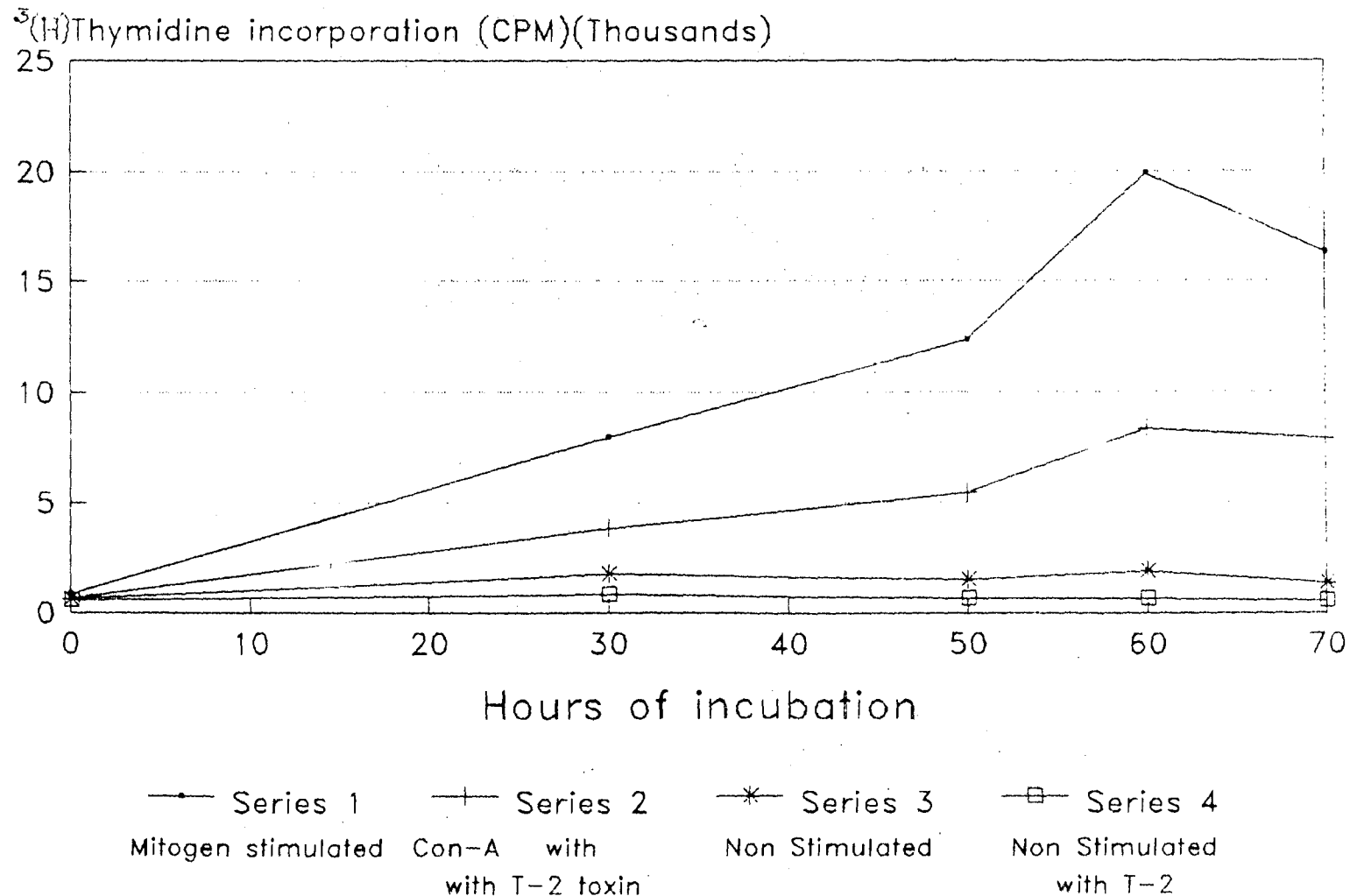
The toxic manifestation of T-2 Toxin in relation to the dose effects (100ng and 100 pg) with the time intervals (7, 14 and 21 days) were studied and summarized in table I to IV.

T-2 toxin treatment at higher concentration (100 ng/ml) showed significant reduction in ^{3}[H] Thymidine incorporation at both the time intervals (7 and 14 days). T-2 toxin treatment with less concentration (100 pg) had little effect on ^{3}[H] thymidine incorporation for all time intervals (7, 14 and 21 days).

7 days:

Administration with single doses of T-2 Toxin in two different concentrations were given to two set of Rats and after 7 days, the spleenic Lymphocyte proliferation was studied both in vivo and in vitro. The study clearly showed that a significant reduction in the proliferation of splenic lymphocytes was observed in the experimental rats.

Rats administered with (100 ng) after 7 days clearly showed significant inhibition in spleenic lymphocyte proliferation both in vivo and in vitro. In vivo the percentage of inhibition of ^{3}(H) Thymidine incorporation was 28.57, 35.70, 43.19, 50.67, 43.53 in 0,30, 50, 60, 70 hrs respectively. However the same lymphocytes were challenged with T-2 Toxin, in vitro, proliferation of spleenic

53

lymphocyte were further reduced. The percentage of inhibition of ^{3}(H) Thymiding incorporation was, 30.57, 76.68, 81.8, 85.78, 86.36 in 0, 30, 50, 60, 70 hrs respectively.

In another set of experiments, rats administered with (100 pg) con. of T-2 Toxin, the _in vivo_ percentage of inhibition of ^{3}(H) Thymidine incarporation was 7.78, 10.58, 12.93, 14.78, 10.94 in 0, 30, 50, 60, 70 hrs respectively. However, the same lymphocyte was challenged with T-2 Toxin _in vitro_, the percentage of inhibition of ^{3}(H) Thymidine incorporation was 12.55, 18.75, 20.11, 26.92, 21.54 in 0, 30, 50, 60, 70 hrs respectively.

The effect of T-2 Toxin on spleenic lymphocyte proliferation was studied in dose effect relationship, _in vivo_ and _in vitro_. At 100 ng con. of T-2 Toxin the inhibition of lymphocyte proliferation was higher than at the lower ocncentration (100 pg) of T-2 Toxin. The inhibition of lymphocyte proliferation was less _in vivo_ than the same lymphocyte challeged _in vitro_. The results of the experiments were shown in Table II&Fig. 2.a & b.

15 days

Rats administered with single doses of two different (100 ng), (100 pg) concentration of T-2 Toxin were given separately to two sets of Rats. After 15 days, the spleenic lymphocyte cells proliferation was studied both _in vivo_ and

54

in vitro. It was clearly showed, that the reduction in proliferation of spleenic lymphocytes was observed in lymphocyte isolated from these rats. The dose effect relation was studied. However, the percentage of inhibition of spleenic lymphocyte proliferation was less than the spleenic lymphocyte isolated from the rats sacrifised at 7 days after treatment. The effect of T-2 Toxin effect on lymphocyte proliferation was decreased with increase time when T-2 Toxin present in the body.

Rats administered with (100 ng) after 14 days, in vivo the percentage of inhibition of ^{3}(H) Thymidine incorporation was 13.30, 22.40, 33.14, 37.48, 30.61 in 0, 30, 50, 60, 70 hrs respectively. However, the same lymphocytes were challenged with T-2 Toxin in vitro, the percentage of inhibition of ^{3}(H) Thymidine incorporation was 24.69, 60.23, 73.79, 78.84, 80.99 in 0, 30, 50, 60, 70 hrs respectively.

In another set of experiments rats administered with (100 pg) con. of T-2 Toxin. In vivo the percentage of inhibition of ^{3}(H) Thymidine incorporation was 6.25, 9.08, 9.54, 9.97, 7.75 in 0, 30, 50, 60, 70 hrs respectively. However, the same lymphocyte challegned in vitro with T-2 Toxin, the in vitro percentage of inhibition of lymphocyte proliferation was 10.01, 17.21, 18.34, 24.86, 17.35 in 0, 30, 50, 60, 70 hrs respectively.

The effect of T-2 Toxin on lymphocyte cell proliferation was studied in dose effect relation in vivo and in vitro. Inhibition of spleenic lymphocyte proliferation was observed in both 100 pg and 100 ng con. of T-2 Toxin. However, 100 pg con. of T-2 Toxin, has little effect. At 100 ng con. of T-2 Toxin,the percentage inhibition was higher than the 100 pg con.of T-2 Toxin effect. The inhibition of lymphocyte proliferation was less in vivo. Then , the same lymphocytes were challenged in vitro. The inhibition of spleenic lymphocyte proliferation was decreased when compared with results of rats sacrifised at 7 days after treatment. The results of the experiments were shown in Table III and fig. 3 a & b.

21 days

Rats administered with single dose of T-2 Toxin with (100 pg) concentration, and after 21 days the splenic lymphocyte cells proliferation were studied, both in vivo and in vitro. It clearely showed, at this concentration, when T-2 Toxin present for prolonged period in the body had a little effect on lymphocyte proliferation. Here percentage of lymphocyte proliferation was comparitively less than the 15 days, and 7 days sacrified animals. In vivo, percentage of inhibition [3](H) Thymidine incorporation was 4.84, 7.75, 8.09, 8.91, 7.14 and the same lymphocytes were challenged with T-2 Toxin in vitro, the percentage of

56

inhibition of ^{3}(H) Thymidine incorporation was 8.21, 16.88, 17.31, 22.90, 15.51 in 0, 30, 50, 60, 70 hrs respectively. The results of the experiment are shown in Table IV and Fig. 4.

Table II Effect of T-2 Toxin on Con-A stimulated spleenic Lymphocytes in vivo and in vitro after 7 days.

^{3}(H) Thysidine incorporation (CPM ± S.D)

Hours of Incubation	Mitogen Stimulated Lymphocyte (Con A 20 µg/ml)	in vivo				in vitro			
		100 pg/ml of T-2 Toxin	% of Change	100 ng/ml of T-2 Toxin	% of Change	100 pg/ml of T-2 Toxin	% of Change	100 ng/ml of T-2 Toxin	% of Change
0	851.25 ±22	785 ±65	7.78	608 ±45	28.57	744.4 ±48	12.55	591 ±53	30.57
30	7970 ±49	7126 ±57	10.58	5124 ±33	35.70	6475 ±42	18.75	1858 .7	76.68
50	12364.75 ±10	10765 ±45	12.93	7024 ±56	43.19	9878 ±39	20.11	2250.33 ±15	81.8
60	19876 ±17	16973 ±52	14.78	9804 41	50.67	14524 ±24	26.92	2825.6 40	85.78
70	16246.25 ±30	14468 ±23	10.94	9174 ±41	43.53	12746 ±30	21.54	2214.6 ±16	86.36

Values represent the mean of± S.D. for three observations

The same results were expressed in fig 2a & b.

58

Table III Effect of T-2 Toxin on Con-A stimulated spleenic Lymphocytes *in vivo* and *in vitro* after 14 days.

^{3}(H) Thymidine incorporation (CPM ± S.D)

Hours of incubation	mitogen stimulated Lymphocyte (Con A 20 µg/ml)	in vivo				in vitro			
		100 pg/ml of T-2 Toxin	% of change	100 ng/ml of T-2 Toxin	% of change	100 pg/ml of T-2 Toxin	% of change	100 ng/ml of T-2 Toxin	% of change
0	851.25 ±22	798 ±51	6.25	738 ±23	13.30	766 ±30	10.01	641 ±18	24.69
30	7970 ±49	7246 ±47	9.08	6184 ±41	22.40	6598 ±32	17.21	3169 ±13	60.23
50	12364.75 ±10	11184 ±30	9.54	8267 ±19	33.14	10096.3 ±25	18.34	3240.64 ±16	73.79
60	19876 ±17	17894 ±32	9.97	12426 ±30	37.48	14934 ±36	24.86	4205 ±59	78.84
70	16246.25 ±30	14986 ±21	7.75	11272 ±48	30.61	13426 ±33	17.35	2543.66 ±15	80.99

Values represent the mean of± S.D. for three observations

The same results were expressed in fig 3a & b.

Table IV   Effect of T-2 Toxin on con-A stimulated spleenic Lymphocytes
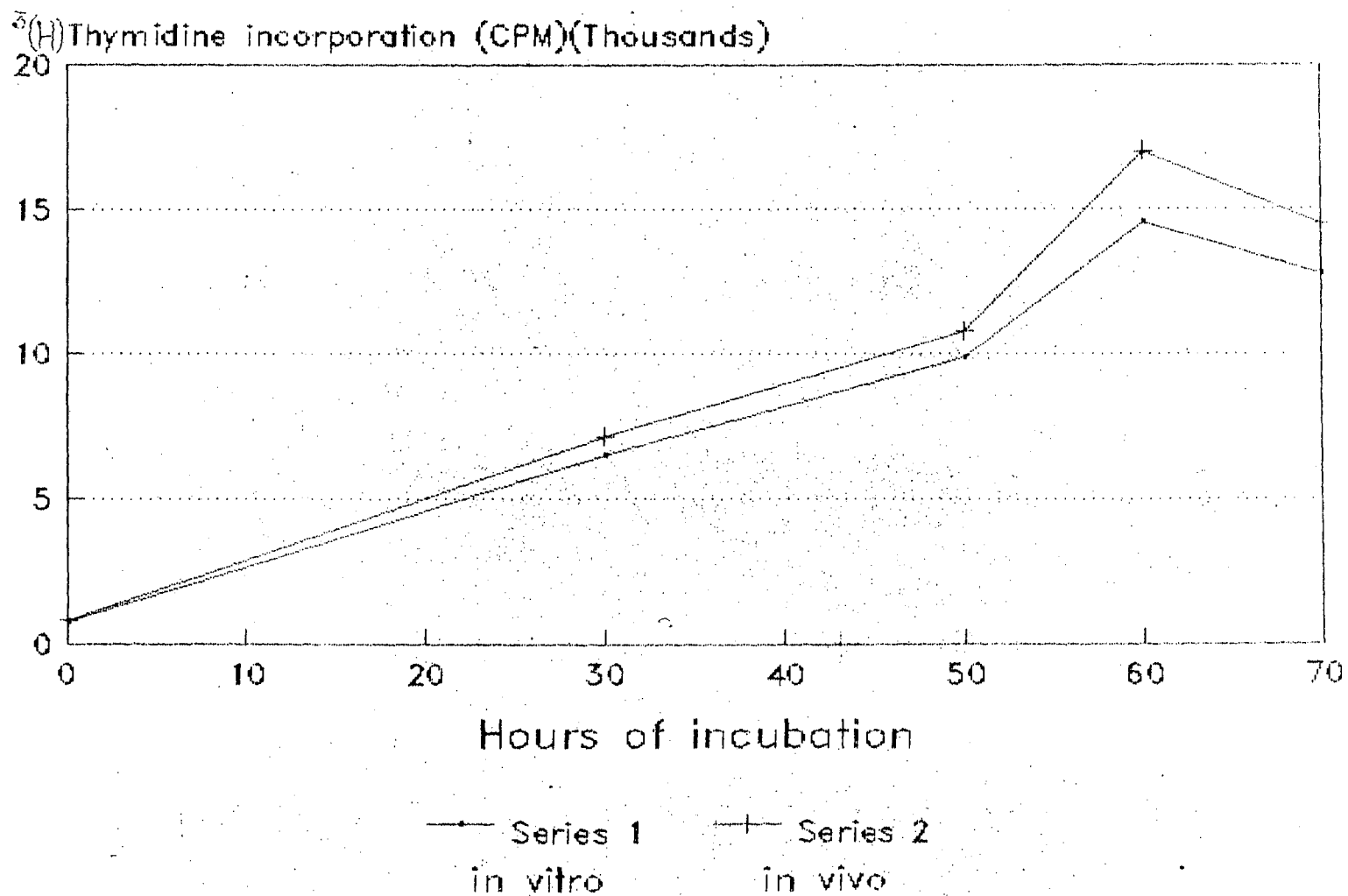in vivo and in vitro After 21 days

	[3H] Thymidine incorporation (CPM ± S.D)				
	in vivo			in vitro	
Hours of in cubation	Mitogen stimulated Lymphocytes (Con A 20 µg/ml)	100 pg/ml of T-2 Toxin	% of change	100 pg/ml of T-2 Toxin	% of change
0	851.25 ±22	810.00 ±39	4.84	781.33 ±33	8.21
30	7970 ±49	7352 ±41	7.75	6624 ±56	16.88
50	12364.75 ±9	11364 ±24	8.09	10224 ±36	17.31
60	19876 ±18	18104 ±13	8.91	15324 ±18	22.90
70	16246.25 ±30	15086 ±35	7.14	12254 ±32	15.51

Values represent the mean of ± S.D. for three observations
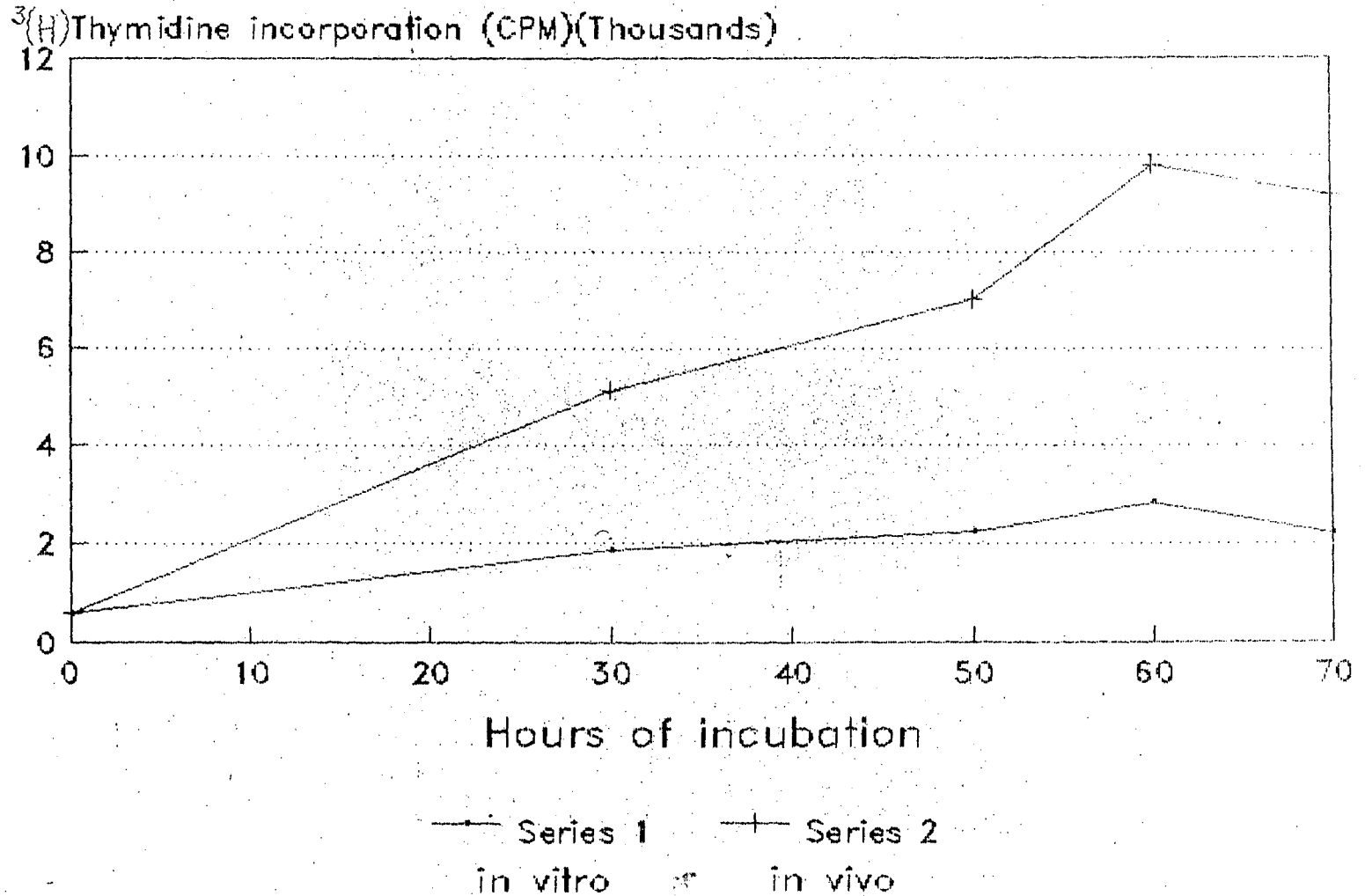
The same results were expressed in figure 4.

Fig. 2A

Effect of T–2 Toxin (100 pg/ml) on Con–A
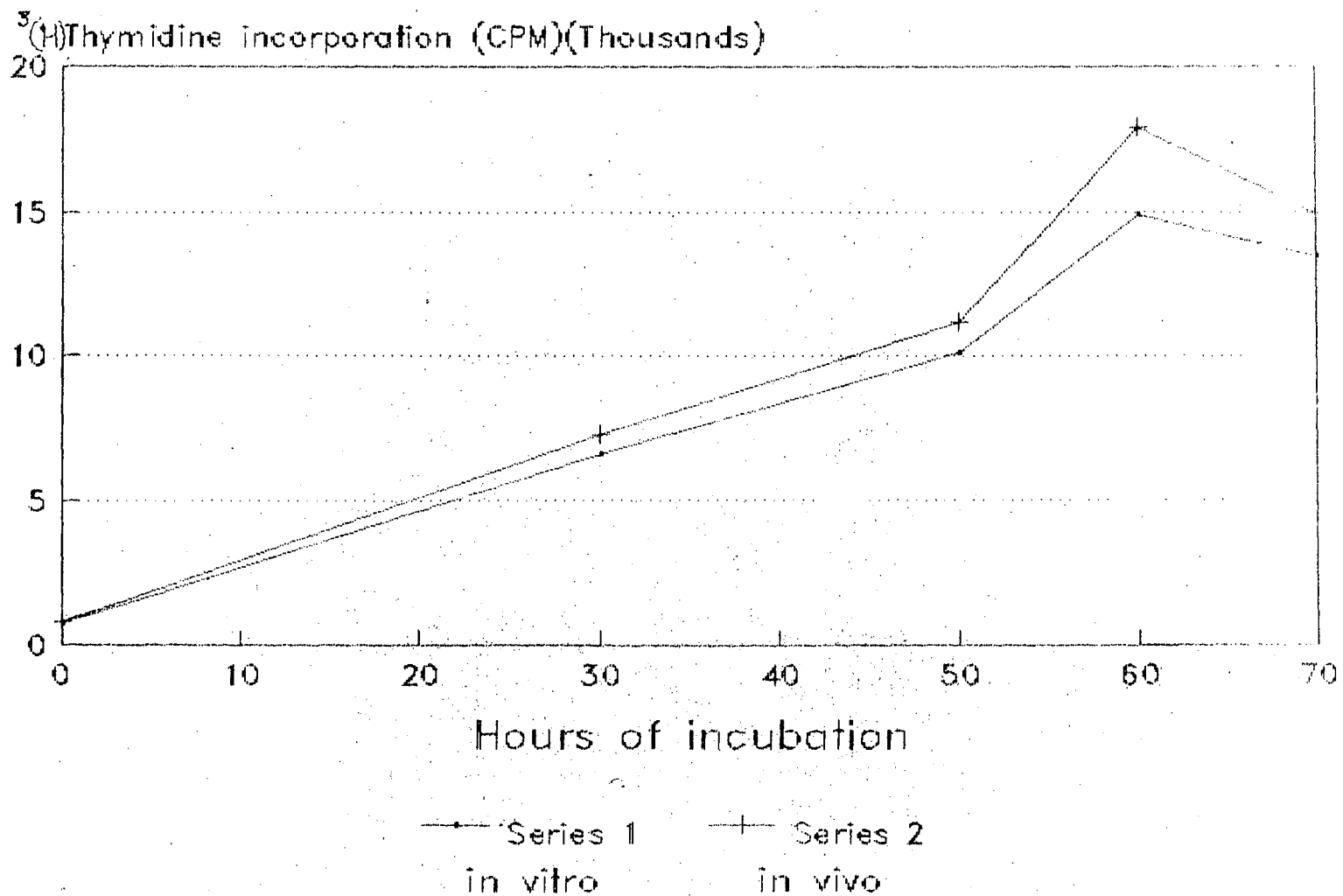Stimulated Lymphocyte after 7 days

^{3}(H)Thymidine incorporation (CPM)(Thousands)

Hours of incubation

Series 1
in vitro

Series 2
in vivo

Fig. 2B

Effect of T-2 Toxin (100 ng/ml) on Con-A
Stimulated Lymphocyte after 7 days

^{3}(H)Thymidine incorporation (CPM)(Thousands)

Hours of incubation

Series 1   Series 2
in vitro   in vivo

Fig. 3A

Effect of T–2 Toxin (100 pg/ml) on Con–A
Stimulated Lymphocyte after 14 days

^{3}(H)Thymidine incorporation (CPM)(Thousands)

Hours of incubation

Series 1   Series 2
in vitro   in vivo

Fig. 3B

Effect of T-2 Toxin (100 ng/ml) on Con-A
Stimulated Lymphocyte after 14 days

^{3}(H)Thymidine incorporation (CPM)(Thousands)

Hours of incubation

Series 1 ---+--- in vitro   Series 2 ---+--- in vivo
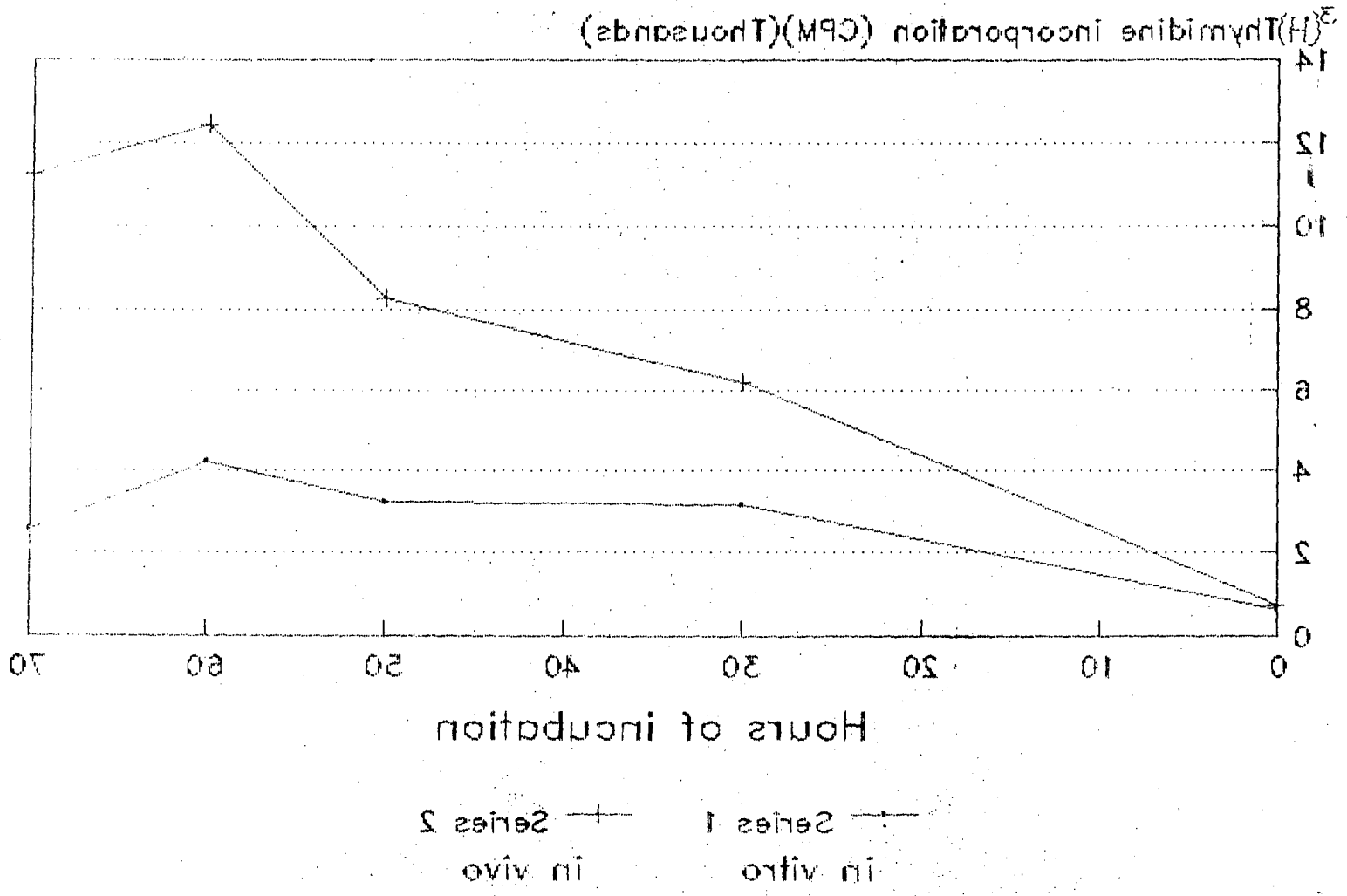
Fig. 4

Effect of T-2 Toxin (100 pg/ml) on Con-A
Stimulated Lymphocyte after 21 days

^{3}(H)Thymidine incorporation (CPM)(Thousands)

Hours of incubation

Series 1   Series 2
in vitro   in vivo
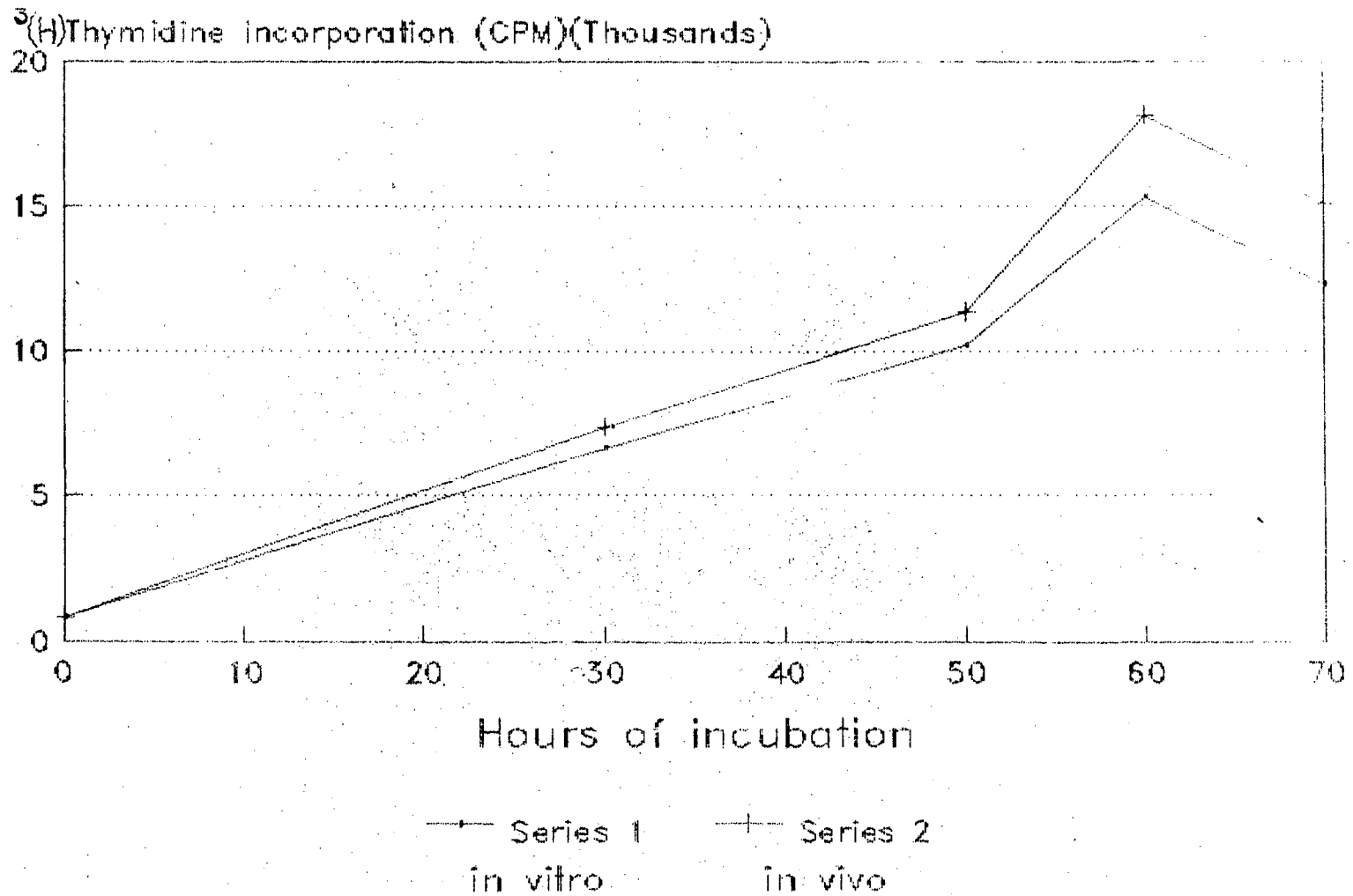
DISCUSSION

Our current knowledge of immuno-suppressive effect of T-2 Toxin on cell mediated and humoral immunity is limited and therefore,it necessitates further investigation of the effect of T-2 Toxin. In vivo and in vitro lymphocyte proliferation, over a reasonable duration of exposure, an attempt to study the effect of T-2 Toxin to repeated challenges, lymphocytes were chosen as the subject. Lymphocyte proliferation has been considered to be an index for in vitro cellular immunity.

The parameters chosen in this study were sensitive indicators of the lymphocyte, reflecting the immological status of the subject (Oppenhein and Schector 1976). This in vitro model is now widely recognised in immunotoxiclogical studies (Bacn 1975) especially in clinical immunology and genetic toxicology. The relevance of immunology along with toxicological evaluation has been stressed by many researchers (76).

In the present study, only DNA synthesis was studied, because replication of DNA is the central controlling mechanism which controls all other cellular processes.

Mitogenic Response of Spleenic Lymphocyte with T-2 Toxin

The present study showed that when con-A stimulated

lymphyocytes cultured with T-2 Toxin, in higher concentration i.e. 100 ng/ml, showed significant inhibition in lymphocyte mitogenesis. At this concentration T-2 Toxin, could manifest its toxicity on lymphocyte mitogenesis. However, at lower concentration (100pg/ml) did not show any significance in mitogenic response. Lafarge Frayssinet et al., (1979) showed that T-2 toxin inhibited PHA-stimulation of splenic lymphoid cells at concentrations of 2-10 ng/ml but enhanced the mitogenic response at concentration between 0.05 and 1 ng/ml. However, there was no greater inhibition of mitogenic responses were observed in the present study. In another study conducted by Guongyossy Irsa et al., (1985), showed that at lower dose 125 pg/ml the minimal toxic effect manifested itself several hours after the introduction of the toxin into the culture.

The present study clearly showed that there was a dose dependent inhibition of con-A stimulated lymphocyte proliferation by T-2 Toxin, (Table I)

Proliferation & Blastogenesis

This study for the first time has evaluated the temporal effect of T-2 toxin in rat over 21 days. Administrating with T-2 Toxin, the pattern of lymphocyte proliferation was studied in vivo and in vitro after different time periods viz 1,2,3 weeks, with two different concentrations namely 100 ng/ml and 100 pg/ml.

The lymphocytes isolated during these periods were evaluted with and without T-2 toxin, to understand the time dependent effect and a subsequent challenging dose effects.

Both at 7 days, (Table II) and 15 days (Table III) after administration of (100 ng/ml) T-2 Toxin in rats, the spleenic lymphocyte proliferation in vivo was inhibited. However, the percentage of inhibition(100 ng of T-2 Toxin) of spleenic lymphocyte proliferation was high in 7 days rather than in 15 days. When the Toxin present in body for long time, its effect on spleenic lymphocyte was reduced. When the same lymphocyte challanged in vitro with toxin, splenic lymphocyte proliferation was further reduced than in vivo.

The study revealed the effect of the toxin after 7 days, (Tab. II) 14 days (Tab III) and 21 days (Tab IV) at a lower concentration (100pg /ml) was little effective on the ^{3}[H] thymidine incorporation of both in vivo and in vitro. In all the three sets of experiments this effect was gradually reduced with increase in time.

This trend was highlighted by observing the result of the third set of experiment (after 21 days of administration) which was having almost similar effect as the control.

Lymphocytes isolated from the experimental rats

63

showed reduction in ^{3}[H] Thymidene incorporation into DNA. However, in vitro the addition of T-2 toxin (i.e. repeated challenge) to same lymphocyte isolated from treated animals showed further inhibition of ^{3}[H] Thymidine incorporation was much more significant. Most importantly, it was noted that the inhibition of the cell proliferation was observed both in vivo and in vitro. The inhibition of lymphocyte proliferation was more in vitro than in vivo

The Parameter chosen in this study is ^{3}[H] Thymidine incorporation into DNA, which is the index for cell proliferation. Lymphocyte proliferation was inhibited by the toxin, which might be the reason why the immunosuppresion occured. Lymphocyte proliferation is very much essential for antibody mediated neutralization of the toxin or for recognition of the same by MHC (Major histocompatibility complex) mediated cytotoxic effects. The T-2 toxin is also known as potent inhibitor of DNA, RNA and protein synthesis. We have shown that in vitro, it inhibited lymphocyte proliferation which might be the better reason for the inability to remove the toxin or to counteract, the effect of the toxin through immune system.

Possible Entry of T-2 Toxin into Lymphocyte

When the cells were incubated with ^{3}(H)T-2 toxin a significant increase in the quantity of T-2 toxin association occcured,during first 30 min. This increased

64

further from 10-16 hrs and decreased after 24 hrs. The longer the contact period the greater the accumulation of the toxin with in the cell (66). The amphipathic behaviour of T-2 Toxin suggested that the cellular hydrophobic sites such as the membranes are the first possible point of interaction (62). Then the toxin may enter into the cell by receptor mediated endocytosis. The extreme sensitivity of lymphoid tissue to T-2 toxin could be explained by the fact that these cells possess only low level of detoxifying enzymes such as Glutathion Transferase.(68)

Effect of T-2 Toxin on Nuclear - Membrane Communication.

It has been suggested that T-2 Toxin can exert its influence on the cell membrane (56). In lymphocyte-antigen interaction after receptor occupancy, a signal must be transmitted from the membrane to the nucleus to have a response from the cell. Similarly, T-2 Toxin could interfere with membrane-nuclear communication may result in an inhibition of the communication between the nucleus and the membrane. At this point T-2 Toxin could interfere with the macromolecular synthesis.(63)

T-2 Toxin and Immunoregulatory Molecules

The mitogens (Con A, PHA) have receptors on surface of both B and T cells. The mitogens are capable of activating T-cells. This was reflected in their ability to stimulate the DNA synthesis. The mitogen activated T-cells

are induced to release humoral factors which had physiological effects. T-cells are required for both inducing antibody sythesis and cell mediate reactions(77). The present study showed that the toxin inhibits the Con A stimulated lymphocyte proliferation by measuring the incorporation of ^{3}(H) Thymidine. Con-A has been shown to induce the release of humoral factors from the thymus lymphocytes which are required for the DNA synthesis in B cells(77). Buening et al. 1987 showed that Con-A and PHA responses were significantly depressed in the toxin administered calves.

SUMMARY

Experiments have been conducted to study the effect
of T-2 Toxin on spleenic lymphocyte proliferation on Con-A
stimulated rat lymphocyte cultures. Criteria employed for
assessing the lymphocyte proliferation in the present study
were based on ^{3}[H] Thymidine incorporation in culture
cells, which were widely accepted in vitro model to evaluate
cell mediated immunity. A brief account of the experiments
conducted and the findings of the present study are
summarised below.

1. Rats were divided in 2 groups. One group was kept as
    the control experiment while the other group was used
    for the test experiments.

2. In control rats the ^{3}[H] Thymidine incorporation into
    Con-A stimulated and non-stimulated splenic lymphocyte
    were studied only in vitro.

3. The test rats administered with a single dose of 100 ng
    and 100 pg (for 200 kg body wt) of the toxin and
    lymphocyte proliferation were studied in vivo and in
    vitro after 7, 14 and 21 days.

4. Lymphocytes isolated from these administered animals
    were cultured and ^{3}[H] thymidine incorporated into DNA
    were chosen as in vivo model.

5. The same lymphocytes isolated from the administered
    rats and challenged with the toxin in vitro were chosen
    as in vitro model.

6. The toxin inhibited ^{3}[H] Thymidine incorporated in both Con-A stimulated and non stimulated lymphocytes.

7. Dose dependent inhibition relations were observed with different T-2 Toxin concentrations.

8. The results were discussed in terms of possible immunosuppressive mechanism of action of the toxin on proliferating lymphocyte population at the cellular level.

9. Biological implications of defective lymphocyte transformation are discussed in the light of cell mediated immunity.

10 Immunoregulatory mechaisms are discussed as the possible antigenic responses to the toxic action.

REFERENCES

*1. C.W. Hesseltine, O.L. Shotwell, M.L. Smith, J.J. Ellis, E.E. Vandegraft, and M.L. Goulden, (1969) Mycologia 60, p 304 and S.K Mishra, H.S.R. Murthy, Currn. Sci. (India) 37, 406.

2. M.Saito, M. Enomoto, T.Tatsuno and K. Uraguchi, Microbial Toxions (1971) (Ed. A. Ciegler, S.Kadis and S.J. Ajl) Vol-VI, p. 299, Academic press.

3. B. Wilson, Microbial Toxins,(1971) (Ed. A. Ciegler, S. Kadis and S.J. Ajl) Vol-VI, p. 489, Academic press.

*4. C.W. Hessltine, E.E. Vandegraft, D.I. Funnell, M.L. Smith, and O.L. Shotwell, (1972) Mycologia 64, 539.

5. A.W. Hayer and B.J. Wilson, (1968) App. microbiol. 16, 1163.

6. J.R. Bamburg and F.M. Strong, Microbial toxins, (1971) (Ed. S. Kadis, A. Ciegler and S.J. Ajl) Vol-VII, p. 207, Academic press.

*7. C.M. Christensen, G.H. Nelson, and C.J. Mirocha (1965) App. Microbiol, 13. 653.

8. Christiansen C.M. (1965). Fungi in cereal grain's and their products pg 9-41 in GN. Wogen (Ed) mycotoxins in foodstuffs M.I.T Press, Cambridge. MA.

*9. H.W. Schroeder; (1963) phytopath•53, 843,

*10. N. James, J. Wilson, and E. Stark, can. J. Res, 24, 224,(1946).

*11. R.J. Bothast, G.H. Adams, E.E. Hat field and E.B. Lancaster, Jr. Dairy Sci, 58, 386, (1975).

*12. Joffe, A.Z. (1974) Toxicity of Fussarium Poae and F. sporotrichioides and its relation to alimentary Toxic aleukia in [I.F.H. Purchase ed], pp. 220-263, Elsevier, Amsterdam.

*13. Ylimari; A., Koponsn, H., Hintikka, E.L., Nummi, M., (1979) Mycroflacrat and occurrance of Fusaricum Toxins in Finish grain. Technical Research Centre of Finland. Publication 21.

14. V. Hietaniemi and J. Kumphlainen, (1991) contents of fusarium toxin in finished and improved grains and feeds food additions and contaminants Vol-8 No. 2 pg 171-182.

*15. Karppanen, E., Rizzo, A., Berg, S., Lindfors, E., and Aho, R., (1985) Fusarium as a problem in Finnish feeds and cereals. Jr. of Agri. Sci. in Finland 57, 195-206.

*16. Tanaka. T., Hasegawa, A., Yamamoto, S., Lee, V.S., Sugiura, Y., and Yeno, Y, (1988) World wide Contamination of cereals by the Fusarium mycotoxins nivalenol, deoxynivalenol and Zearalenone, Jr. of Agri food chemistry·36, 979-983.

17. Ramesh V. Bhat, Sashider Beeds, Y. Ramakrishna K.L. Munshi, (1989) outbreak of Trichothecene mycotoxicoses associated with consumption of mould-damaged wheat products in Kashmir Valley, India. Lancet, Janunary 7, pg 35-37.

18. Fun Sun Chu, (1984) Jr. of food protection, Vol-47, July pg 562-569.

19. Titans. S.L.Fan, Guang S. Zhang and F.S. Chu, An. Jr. of food protection· Vol-47, No. 12 pg 964-967 (Dec 1984).

*20. J.A. Winstead and R.J. Suhadolnik (1960) J. Am. Chem. Soc. 82, 1644.

*21. P.M. Scott, M.A. Merrien and J. Polonsky, (1976) experimentia·32, 140.

*22. O.R. Rodig, (1971) Chem. Commun. 1553.

*23. K. Nitta, Y. Yamamota, T. inour and T. Hyodo, Chem. Pharm. Bull. 14, 363, (1966).

24. A. Rhodes, G.A. Somerrfield and M.P. McGonagle, (1963) Biochem. J. 88, 349.

25. M.T. Lin, D.P.H. Hsieh, R.C. Yao-and J.A. Donkersloot, (1973) Biochemistry 12, 5167.

*26. Jones. E.P.H. and Lowe, G. (1960) J. Chem. Soc. p. 3959.

*27. Achilladelis, B., Adams, P.M., and Hanson, J.R. (1970) Chem. Commun, p. 511.

*28. Hohn, T.M., and F.L. Van Middlesworth (1986), Arch. Biochem. Biophys 251: pg 756:761.

29. Susan, P. McCormick, Scott L. Taylor,k Ronald D, Plattner, and Marian Beremand.(1990 March) Bioconversion of possible T-2 Toxin precursors by a Mutant strain of F. Sporotrichoridy RRL 3299. App. and Env. Microbia p.2702-706.

30. Beremand M.N (1987), App. Env. Microbiol. 53:1855-1859.

31. Anne, E. Desjardins, Ronald D. Plattnor, and frank, Vanniddlesworth (May 1986) App. and Env. Microbol 1193-1197.

32. LLoyd B. Bullerman, Lisal. Schroeder and Kun-Young Park. Jr. of Food protection: 47 (8) pg 637-646.

*33. Sanderl. T.H., N.D. Davis, and V.L. Diener, (1968), J. Amer. oil chem. Soc, 45:683-685.

34. Robert F.Fricks, (1990) Clinical Toxicology 28(4), 421-431.

35. Toxicology Biochemistry and pathology of mycotoxins, Edited by Kenji uragunchi, Miko Ymazaki,John Wiley & Sons, page 53-54.

36. Thomas D, Wyllie and Lawrence G Morehouse, Mycotoxic fungi, mycotoxins, mycotoxicoses and encyclopedic Hand Book Vol-3, page 161-171.

*37 V.A. Tutelyan and L.V. Kravchenko, Mycotoxins, Medicine, Moscow, 1985, pp 319.

38. P.K.C Chan, P.A. centry, (1984) LD 50 Values and serum Biochemical changes T-2 Toxin in Rats. Toxicology App. Pharm. 73, 402-410.

39. J.G. Pace, (1983) Effect of T-2 Toxin mycotoxin on Rat liver mitochondria electron transport system, Toxicon 21 pg. 675.

40. S.K. Suneja, D.S. Wagle and C.Ram, Effects of T-2 Toxin in gavage on the synthesis and contents of Rat liver macromolecules. Fd. Chem.Toxicol(1987) Vol. 25, No. 5, pg 387-392.

41. Lydia V. Kravchenko, V.A. Tutelyan, A.V. Vasilyev, A.E. Kranauska and Ludimela I. Avren Yena. (1986) Biochemical changes in subacute mycotoxicoses induced by T-2 Toxin in Rats. Toxicology.42 pg. 77-83.

42. Y. Ueno, (1985) Toxicology of mycotoxins. Crit. Rev. of Toxicology.Vol. 74(2) pg 99-132.

43. R. Yoran, R. More, A. Eldor, and B. Yagen. (1984) The effect of T-2 Toxin on Human platelet. Toxic App. pherm·73, pg. 210-217.

44. P.K.C. Chan and P.A. centry, inhibition of Bovine platelet function by T-2 Toxin, HT-2 Toxin, diacotoxy scre Pinol and Deoxynivalenol (1984), Vol 22, No. 81 pg 643-648.

45. Rath. Sega I Lana mico-Gold heag. Auram Z. Joffe and Boris Yagan (1983) Trichottecene induced haemolysis I. The hemolytic activity of T-2 Toxin. Toxic app. pharm. 70 343-349.

*46. Y. Ueno and H. Malimoto (1973) Inactivation of some thiol enzymes by trichothecene mycotoxins from fusarium sp. Chem. Pharm. Bull. 23, p. 2439.

47. L.V. Krachenko, S.T. Khuylya, L.T. Avranyena. I.A. morozov and V.A. Tutelyon,(1983) Effect of T-2 Toxin on the ulttra structure and activity of organelle specefic enzymes of some rat tissue cytologia No 11 (1983) 1264.

48. S.K. Sunga, D.S. Wagle, G.C. Ram, Toxicon (1984) Effect of orgal administrationof T-2 Toxin on Glutathione shuttle microsomal reductase and lipid peroxdation.

49. M. Ohat, H. Matsumota, K. ishii, and Y. Urna, (1978) Metabolison of Trichothecene mycotoxins II. Substrate specificity of microsomal deacetylation of Trichothecenes Jr. Biochem 84 pg. 697-706.

50. M. Ohat, Kenji and Y. Ueno (1977) Metabolism of Trichothecenes mycotoxins. 1 microsomal Deacetylation of T-2 Toxin in animal tissue. Jr. Biochemistry. (1977) Vol. 82 pg 1591-1592.

*51. M. Ohta and Y. Ueno, (1976) Pros. Japan. Ass. Mycotixicol. (Japanese) 2, pg. 134.

52. R. Cooray and Lindahl-Kiessling, (1987) Effect of T-2 Toxin on the spontaneous antibody secreting cells and other non-lymphoid cells in the murine spleen, Food and chemical toxicology Vol. 25(1) pg 25-29.

53. Lafarge-Frayssinet, G. Lespinats, P. Lafont, Foloi-ilier, S. Mousset, Y. Rosensteen and C. Frayssinet, (1979) immunosuppressive effects of Fusarium Extract and Trichothecenes: Blastogenic response of murine splenic and Thymic cells to mitogens (40439), pro. of the Soc. For. Exp. Biol and Med.Vol. 160 pg 302-311.

54. V. Jagadeesan, C. Rukmini, malini Vijayaraghavan and P.C. Tulphle, (1982) immne studies with T-2 Toxin. Effects of feeding and with drawal in Monkeys, Food and chem. Toxical·Vol 20. pg 83-87.

55. R. Yarom, Y. Shiriman, R. more, I Ginsburg and R. Borinski and B. Yagen, T-2 Toxin Effect on Bacterial infection and Leukocyte functions. Toxic and app. pharmocology (1984) 75, 60-68.

56. Ueno, Y. Nakajima, M. Sakai K., Ishii K., Sata, N., and Shimada, N. (1973) comparitive Toxicology of Trichottecene mycotoxins: inhibition of Protein synthesis in animal cells. Jr. Biochemistry 74: 285-296.

57. C. Lafarge-Frayssint, F. Decloitre, S. Mousset, M. Martin and C. Frayssint, Induction of Single strands by T-2 Toxin: Mutation Research (1981) 88: pg 115-123.

58. Franciso-Javier plasencia and Yvonne Rosenstein, effect of in vivo administration of T-2 Toxin on Peritoned murine macrophages, Toxicon (1990) Vol- 28(5) pg 559-567.

59. Rasenstein, Y., Kretschmer, R. and Lafagre-Frayssinet, C. (1981) Effect of Fusarium T-2 Toxin and diacetoxyscripenol on murine T-dependent immune response, immunology 44, 555-560.

60. Hornal Foetu, Douglas D. Mann, Gerald, m. buening, Barbara Shook and Gary, D. Osweiler. Effect of T-2 Toxin on Bovine immune system infection and immunity (1982) June, pg 1249-1252.

61. Fairhust, S., Maxwell, S,A Scawim, J.W and Swanston. Dow (1987) Skin effects of Trichothecenes and their amelioration by decontaminantion Toxicology 46. pg 307-319.

62. Gyongyossy-issa M.I.C and khachatourions G.G. Biophy, Biochem. Acta, (1984) 803 pg 197-202.

63. M.I.C. Gyongyossy-Issa and G.G. Khachatourians, (1985) interaction of T-2 Toxin and murine lymphocyte and demonstration of a threshold effect on macromolecule synthesis Biophysica Biochemica acta 844, pg. 167-173.

64. Kelvin E. Smith and Michal (1975) Common inhibition of initiation level of Eucariolc protein synthesis by T-2 Toxin FEBS letter Vol-50. Jan pg 8-12.

65. Eric Cundliffe Michael Cannon and juliam Davies (1974) Mechanism of inhibition of Eucariotic protein synthesis by Trichothecene fungal Toxins P.A.N.S Vol. 71, No. 1, pg. 30-31.

66. J.M. Porcher, C. Dahal, C. Lafarge-Frayssinet, F.S. Chu and C. Frayssinet, Fd. Chem, Toxicol (1988) Vol-261, No. 7 pg 587-593.

67. Rostenstien Y. Laefarga-Frayssinetcelespinates G.-Oisither, E. Lafont, P. and Frayssinet (1979) Immunosuppressive active of Fusarium toxins : effects on antibody synthesis and skingrafts of curde extracts. T-2 Toxin and Diactoxyscripenil, immunology, 36, pg. 111.

68. Y-Rosenstein and C. Lafarge-Frayssinet (1983) inhibitory effects of Fusarium T-2 Toxin in Lymphoid DNA and protein synthesis Toxicology and Apploid pharmocol*70, 283-288.

69. Narman J. Kleimon Daniel, L. Friedman and Giovammi D. Sabata (1984) Preparation of single cell suspension from lymphoid organs. Methods in Enzymology, Vol. 108. pg. 43-49. Academic Press.

70. Waithe W. E & Hirschhorn (1973) In: Cellular Immunology edition (Ed D.M. Weif) Black Well scientific publication pg. 25.

71. Kossis D.J. (1972) satistics , John Wiley of sons Inc. New York.

72. Khannan (1979) Leucocytic responses as bioindicators of DDT and Iomex toxicity (unpublished data) J.N.U.

73. S.K. Bohidar (1988) Some immune responses of leucocytes to cadmium-Thionein, J.N.U. Pg. 50-60.

74. Oppenhiten J.I. and Schector B. (1976) In manual of clinical immunology (eds. N.R. Rose and i. Friedman) American Society for Microbiology, Washington D.C. 081 94.

76. K. Miller (1981) Relevence of immunology to toxicology Fd. Cosmet. Toxicol. 19, 125.

77. James Watson, Ruth Epstein, Iloha Nakoinz and Peter Ralph (1973) The role of Humoral factors in initiation of in vitro Priming immuno response Jr. of immonology, Vol. 110 (1).

*78. Buening G.M. Mann, D.D., Hook B. and Oswiler G.D. (1982) The effect of T-2 Toxin on the bovine immune system: Cellular factors, Vet. Immun. Immunopath. 3, pg. 411.

----

* Original not seen.