IN VIVO AND IN VITRO STUDY OF MURINE CYTOKINE GENE EXPRESSION VIA RNA AND PROTEIN ANALYSIS

BY

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To my beloved Tim,

my dear parents

and dear grandmother

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LIST OF ABBREVIATIONS

Ag	antigen
Anti-CD3	anti-CD3 monoclonal antibody 145-2C11
APC	antigen presenting cell
BCKG	background
bp	base pair
CK	cytokine
Con A	concanavalin A
cpm	counts per minute
CSIF	cytokine synthesis inhibitory factor
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	Thymidine-5' triphosphate
dpm	disintegrations per minute
E. coli	Escherichia coli
ETOH	ethanol
FCS	fetal calf serum
hr	hour
IL-2	interleukin 2
IL-4	interleukin 4
IFNr	interferon-gamma
i.p.	intraperitoneal

i.v.	intravenous
kb	kilobase
LAK	lymphokine activated killer cell
LK	lymphokine
LMW-BCGF	low molecular weight B cell growth factor
LPR	late phase response
mAb	monoclonal antibody
MHC	major histocompatibility complex
min	minute
mCAS	mouse Con A supernatant
Mr	relative molecular mass
NEAA	nonessential amino acids
OA	ovalbumin
OA-POL	glutaraldehyde-polymerized ovalbumin
OD	optical density
PCR	polymerase chain reaction
PMA	phorbol myristate acetate
RCAS	rat Con A supernatant
RE	restriction endonuclease
rIL-4	recombinant interleukin 4
rpm	revolutions per minute
RPMI	cell culture medium
SD	standard deviation
STD	standard

sec	second
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
U	unit
UV	ultraviolet

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ABSTRACT

Cytokines (CK) as a group of essential cell-cell communication mediators, as well as important stimulatory and immune regulatory molecules, have been studied extensively over the past 10 to 15 years. However, several aspects of CK genes and regulation of CK gene expression still largely remain blank. This study has focused on the regulation of expression of CK genes induced by extracellular stimuli. The expression of three CKs IL-4, IFNr and IL-2, which play important roles in allergic immune responses, was investigated.

Using anti-CD3 monoclonal antibody (mAb, 145-2C11) or antigen (Ag) ovalbumin (OA, allergen) as T cell activation stimulators, the CK gene expression was assessed at the mRNA level by Northern, dot and cell blotting and at the protein level by CK specific bioassays. Various in vivo and in vitro stimulations were employed for this purpose.

The results have revealed some well characterized patterns of mRNA expression and secretion of the three functional CK proteins. Following administration of anti-CD3 mAb in vivo in naive mice, a rapid and transient IL-4 and IFNr gene expression was induced, peaking at 1 hr. The functional IL-4 and IFNr protein could be detected between 45 min to 3 hr after stimulation. After in vitro stimulation

of spleen cells with immobilized anti-CD3, transient IL-4 and IFNr gene expression was detected later, at 13 hr. The protein secretion for the three CKs peaked from 12 to 18 hr. Mice stimulated with antigen displayed a more complex CK gene expression pattern. Taken together, these results provide evidence for the existence of mature IL-4 secreting T cells in vivo in the unstimulated lymphocyte pool.

Conclusions of the study include (i) in vivo administration of anti-CD3 mAb to naive mice induces a rapid, transient and synchronous expression of IL-4 and IFNr genes; (ii) the IL-4 and IFNr mRNAs are readily translated to biologically active proteins; (iii) IL-2 gene expression is not affected by anti-CD3 stimulation in vivo and no functional protein detectable in the bioassay with mRNA synthesis; (iv) differential regulation of IL-2 gene and IL-4, IFNr genes can be observed with in vivo stimulation of anti-CD3; (v) in vitro stimulation of naive spleen cells with anti-CD3 induces a late, transient and synchronous expression of IL-4 and IFNr genes, with their functional proteins translated; (vi) the study has provided evidence for mature IL-4 secreting T cell presence in vivo.

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INTRODUCTION

T cell-derived lymphokines and other cytokines have been studied extensively in the past 10 to 15 years in terms of their physico-chemical properties, biological functions and receptors. The corresponding genes have been cloned, their nucleotide sequences and the deduced amino acid sequences have been determined. However, studies on regulation of expression of various CK genes in response to extracellular stimuli have only begun in recent years. Much is unknown in this area.

This introduction gives a brief overview of the current study and progress in the areas of cytokines, T cell activation and early events, the study of T cell-derived CK gene expression and detection methods, the known mechanisms of signal transduction in CK gene activation and the role of some CKs in allergic immune responses.

Cytokines

(i) Definition

Cytokines represent a group of essential soluble transmitters of cell-to-cell communication *in vivo* (1). They were first discovered to be produced by mitogen or antigen-activated lymphocytes in 1969

and were termed lymphokine (LK) originally, reflecting the belief that they were produced only by lymphocytes (2). Subsequently, it was realized that non-lymphocytic cells such as macrophages, keratinocytes, fibroblasts and many transformed cell lines could also produce factors with similar functions as LKs. Therefore, together with these factors, LKs were termed cytokines (CK) as a class (3).

(ii) Current views of CK functions

Most CKs are produced by more than one cell type upon activation and exert multiple biological activities on more than one target cell type. In addition to many other functions, CKs are critical in the regulation of immune responses. The conventional thought of CK function was initially focused on their providing amplification and differentiation signals for the generation of the effector phase of immune responses (4). Now, it is becoming increasingly clear that CKs may interact to downregulate immune responses. For example, IL-4 has been reported to counteract the IL-2 helper effect on Ag-activated B cells to secret IgM and on induction of lymphokine activated killer cells (LAK) in humans (5, 6). IFNr acts to stimulate some cell types and inhibit others. CKs may regulate the synthesis of one another (32, 33). In addition, CKs may also be involved in tolerance induction: the absence of cytokines following

antigenic stimulation to the immune system has been suggested to be critical in the induction of tolerance. In a model of adult tolerance to alloantigen established by Dallman et al, the altered regulation of the IL-2 pathway and IL-2 deficit may contribute critically to adult tolerance induction (4, 34, 35).

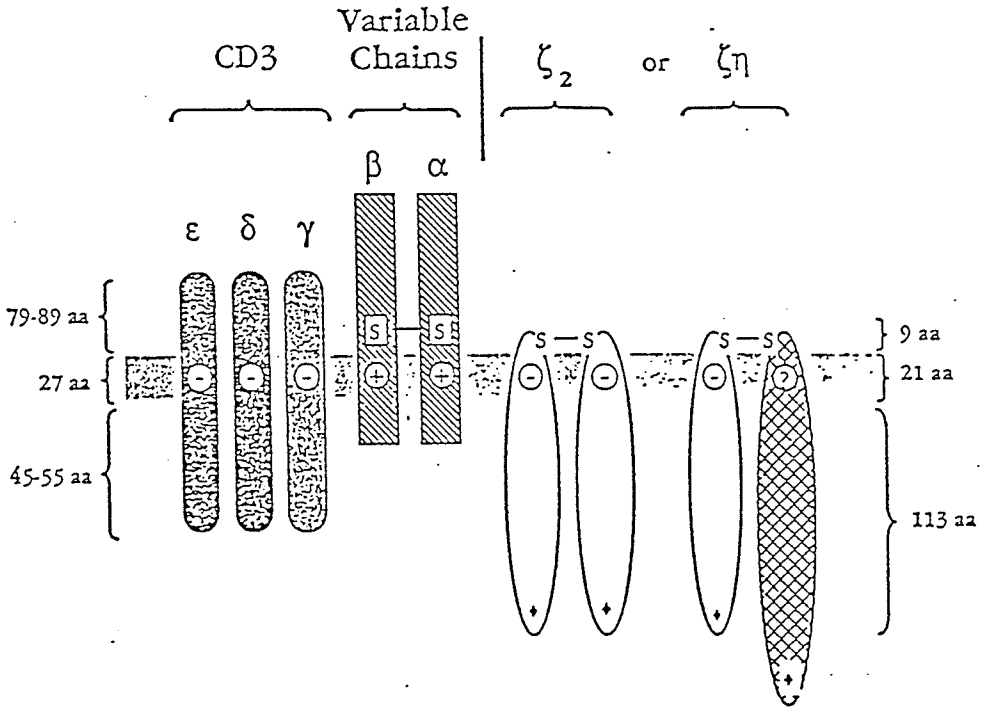
(iii) Recombinant DNA technology in the CK gene study

As with other fields in bio-scientific research, the advent of recombinant DNA technology has also brought the study of CKs into a new era. Many of the CK genes have been isolated and their products become available in pure form (1). Molecular cloning and characterization of various CK genes have provided an opportunity for studying the mechanisms by which CK genes are induced in response to extracellular stimuli and for revealing the relationships between the structure and function of the CKs. The availability of these cloned CK genes made it possible to analyze changes in CK mRNA levels under various conditions; to elucidate the structure of the DNA segments possibly involved in CK gene regulation; to identify functional cis-acting DNA sequences and trans-acting regulatory factors required for CK gene regulation (1).

T cell activation and early gene activation events

There are several means of stimulation which result in activation of resting T cells: Ag specific stimulation, lectin (mitogen) polyclonal stimulation and mAb mimicking stimulation (polyclonal) (36). In vivo, T cells are thought to be activated through the TCR mediated specific recognition of Ag peptide in the context of MHC (major histocompatibility complex) molecules on the surface of antigen presenting cells (APC). This activation process also requires a second signal, usually IL-1 provided by the APC (37). In vitro, T cells can be activated by various agents such as mitogens (Con A) and mAbs against TCR (CD3 or Ti chains, for T-cell idiotype) (1, 38, 39). The current understanding of the TCR complex structure is demonstrated in Fig I. The TCR is divided into 3 groups of subunits: (i) variable chain components, an Ig-like structure composed of α and β chains; (ii) The CD3 complex, composed of 3 proteins γ, δ and ϵ; (iii) ξ and η chains responsible for signal transduction (38).

During activation processes mediated through the TCR, a series of early biochemical events and early gene activation events takes place within the first minutes and hours after the contact of processed Ag-MHC complex with the TCR. Over 100 genes are activated during this process (40). The timing of these events and a list of these genes are shown in Fig.II and Table I (40). It can be seen that proto-oncogenes c-myc, c-fos and T cell-derived CKs IL-2, IL-2R, IL-3, IL-4, IL-5, IL-6 genes are transcribed very early on



⊖, ⊕ Charged amino acid(s) in the transmembrane region
(? indicates unknown)

♦ Homology to a consensus nucleotide-binding sequence

Figure 1 Schematic diagram of the cell surface TCR.

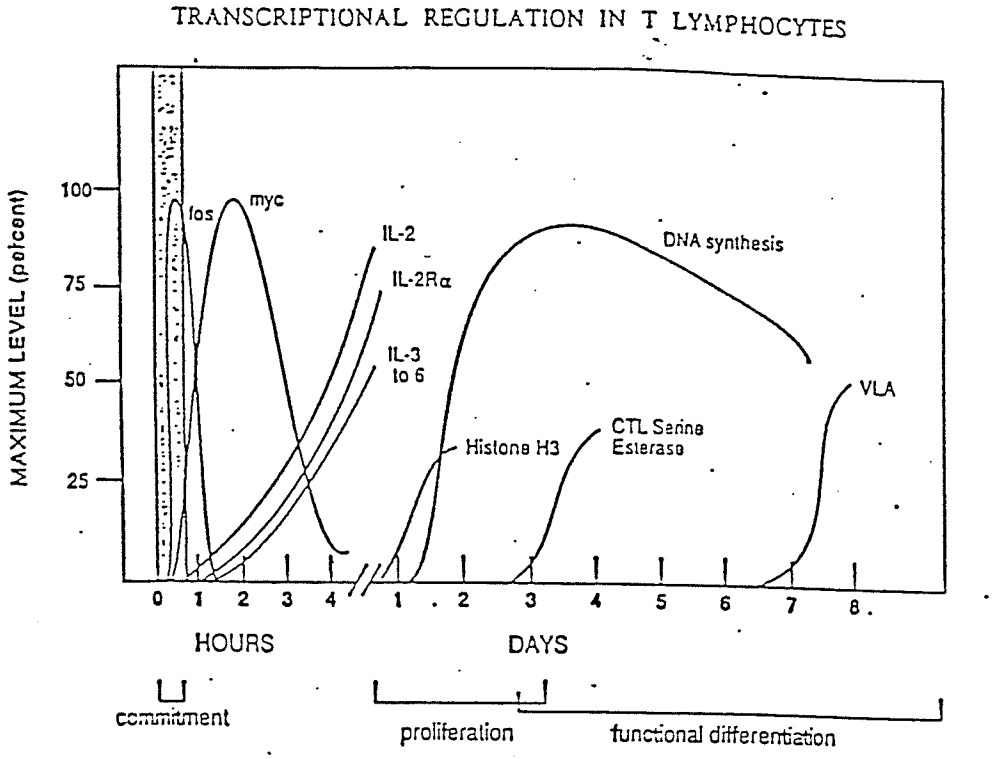


Figure I I Sequences of events initiated in a hypothetical T lymphocyte after antigen activation. Commitment refers to the time after which withdrawal of the initial stimulus does not significantly interfere with proliferation. Only about 50% of T cells become committed in the first 30 minutes. While the early events are probably common to most T cells the late events are characteristic of specific functional types of T cells. Whether a T cell is committed to a particular immunologic function at the time of activation, or can assume different functions as a result of the activation process is not clear, however the CD4 and CD8 markers do not appear to change.

Table I T lymphocyte activation molecules

Immediate Name	Time	Location	Ratio (A/NA)
c-fos*	15 mins.	N	<100
Act-2*	15	S	<10
NF-AT	20	N	~50
c-myc	30	N	20
NF-kB	30	N/C	>10
pAT-225*	30	?	?
pAT-127*	30	?	?
pAT-237*	30	?	?
pAT-251*	30	?	?

Early Name	Time	Location	Ratio (A/NA)
γ interferon*	30 mins.	S	>100
IL-2*	45	S	>1000
bcl-2*	<1 hr	C?	20
TCA-3*	<1	S?	>100
pBK791*	1	?	>10
pBK642*	1	?	>50
ODC*	1	C	10
c-abl*	1	CM and C	~10
SAM decarboxylase*	1	C	5
Insulin receptor*	1	CM	3
p56 lck*	1	CM	1/4
pAT-129*	1	?	?
pAT-243*	1	?	?
HIV-1*	<2	—	>10
TGF-β*	<2	S	>10
actin*	2	C	3
pAT-464*	2	S	?
pAT-744*	2	S	?
IL-2 receptor α*	2	CM	>50
IL-3*	1–2	S	>100
Lymphotoxin*	1–3	S	>100
Ea1	3	CM	>10
Ea2	3	CM	>10
Ea3	3	CM	>10
proenkephalin*	2–4	S	>20
rpt-1*	<4	N	1/5
4F2*	4	CM	?
p28	4	CM	?
pAT-140L*	4	?	?
pAT-620*	4	?	?
pAT-140S*	4½	?	?
pAT-363*	4½	?	?
cyclin*	4–6	C	>10
SECT*	<6	C	370
IL-4*	<6	S	>100
IL-5*	<6	S	>100
IL-6*	<6	S	>100
c-myb*	16	N	100
transferrin*	6–8	S	?
T305*	8	CM	?
L35	12	CM	?
L36	12	CM	?
Transferrin receptor*	14	CM	5
MLR3	18	CM	?
TLISA1*	18–24	CM	?

Table I (continued)

Early Name	Time	Location	Ratio (A/NA)
Glucocorticoid receptor*	20	N	5–6
GM-CSF*	<20	S	?
Galactosyltransferase*	21	CM	12
OK-T10	24	CM	?
AIM	24?	CM	>10
MALA-1	24	CM	10
Neuraminadase*	24–48	C	5
Histone H3*	24–48	N	>10
HIVEN-86A	<48	N	10

Late Name	Time	Location	Ratio (A/NA)
Ta_1	2 days	CM	?
Tp 103	2	CM	?
CTLA-1, Granzyme B*	2	?	>100
CTLA-4*	2–3	CM	>100
HLA-DR*	3–5	CM	10
RANTES*	3–5	S	10
GP-26	3–5	CM	>10
VLA-4	4	CM	>100
HF*, Granzyme A*, CTLA-3	<5	?	>100
CTLA-2	<5	?	>100
CCPII*	<5	?	>100
519*	5	N	>50
Act 1	5–6	CM	>100
LDA-1	9	CM	?
VLA-1*	7–14	CM	>100
VLA-2	7–14	CM	?
VLA-3, VLA-5	10–14	CM	?

The categories are based on an analogy to viral gene expression. Immediate events are independent of protein synthesis; early events require protein synthesis (data not always available) but precede cell division, and late events occur after cell division. Time refers to the earliest time that the molecule can be detected or that an increase could be detected. A/NA refers to the relative levels in activated versus non-activated cells at peak induction. In some cases this data was not available since only fluorescent intensity was measured.

Location refers to the location of the molecule within the cells S, secreted; CM, cell membrane; C, cytoplasmic; N, nuclear.

*Indicates genes that have been cloned.

during the T cell activation process. Late events of Ag specific T cell proliferation and differentiation into specific immune effector cells take place at a time significantly after the Ag contact with the TCR.

An overview of the current study of CK gene expression

(i) The known characteristic features of CK gene expression

The majority of CK genes are not constitutively expressed (4) but rather are transiently expressed following cellular activation (1, 4). This transient nature in the CK gene expression is considered to be important in controlling the cell proliferation, differentiation and function. Under certain circumstances, overproduction of the CKs may result in dysregulation or dysfunction of cells (1). Therefore, the expression of CK genes appears to be strictly regulated by restricted types of cells and inducers (1, 4).

The expression is controlled at transcriptional and post-transcriptional levels. Gene expression of a given CK is also inevitably influenced by other CKs, making the understanding of CK function a highly complex undertaking (1). For example, it is hypothesized that the CKs produced by two different types of mouse T helper clones Th1 and Th2 (see later) can exert mutual inhibitory

effects of each other in vivo. IFNr produced by Th1 clones and IL-4 by Th 2 have been demonstrated to inhibit the synthesis of one another in vitro (33, 81). CSIF (IL-10, see later) produced by Th 2 clones can inhibit the synthesis of IFNr and other CKs produced by stimulated Th 1 clones (33). A "cytokine network" has also been found in human diploid fibroblasts: an enhancing and inhibitory network interaction of tumor necrosis factor (TNF), IFNβ_2, IL-1 and platelet-derived growth factor (PDGF) in IFNβ_2 mRNA expression (41).

(ii) Problems in analyzing CK gene expression

These features of CK gene expression result in some difficulties in the gene expression study in the following aspects : Firstly, due to the transient nature of CK gene transcription, the tissue must be sampled at frequent intervals during the period of interest. Secondly, the post-transcriptional control of the CK gene expression confers on the cytokine mRNA a relatively short half-life compared to mRNA such as β-globin mRNA. It has been found that many CK mRNAs contain AU-rich sequences at the 3'untranslated region, which give rise to an extremely short half-life on the RNA (42, 43). Finally, since certain CK genes are expressed at low levels and frequently by only a few cells , it is necessary to use highly sensitive and specific detection techniques (1, 4).

(iii) Detection methods for CK gene expression

A wide range of detection methods are currently being used in studies of CK gene expression (4, 44), including Northern blot and dot blot hybridization, polymerase chain reaction (PCR), in situ hybridization, ribonuclease protection assays, bioassays and immunoassays. Choice of method to be used in the analysis of CK genes will depend on many factors, including the amount of tissue available and the level of the expression of the CK genes to be studied (4).

Northern and dot blot hybridization techniques are considered to be the most direct and widely used methods for determination of CK mRNA profile. They provide specific, semi-quantitative (Northern) and quantitative (dot blot) data. They can become more sensitive when using high specific activity probes obtained by random primed labeling of cDNA inserts (45) or in vitro transcription of cDNA with phage RNA polymerases (46); using dextran sulfate or polyethylene glycol in hybridization mixture as a volume excluder (48, 49); using nylon membranes instead of nitrocellulose membranes and cross linking the nucleic acids to the membrane by UV irradiation (47); and using efficient and reproducible methods for transferring RNA to the membrane (47). Northern blotting is a widely used technique for the analysis of gene expression (44), particularly for CKs expressed at moderate to high abundance.

The PCR technique provides the most sensitive means of detecting gene expresion. It is of particular use when limiting amounts of tissue are available, or if only rare cells express the gene of interest and the specific mRNA is expressed at low abundance (44). In situ hybridization makes it possible to localize the gene expression to a single cell level (44), approaching the CK gene analysis most elegantly among other detecting methods. However, there are difficulties associated with the use of this technique which prevent its widespread application.

The ribonuclease protection assay is an extremely sensitive and precise method for quantitative mRNA detection due to the protective and sensitive nature of the assay itself. It has been used in CK mRNA studies in transplantation models (50) among others.

As a complementary approach to determining CK gene expression levels, bioassays and immunoassays are highly sensitive in detecting CK protein production. This is important in any evaluation of CK gene expression as mRNA synthesis does not necessarily reflect production of biologically active CK. For example, TGF-β (transforming growth factor beta) mRNA is expressed in both stimulated and unstimulated monocytes, but TGF-β peptide is only synthesized in activated macrophages (21). Therefore, CK mRNA production in itself can not be assumed to be equivalent to the synthesis and release of biologi-

cally active CK. However, all bioassays have the limitation that they only measure the net difference between the amount of CK released in the culture supernatant and the amount of CK consumed by the cells, bound to receptors or natural antagonists (44). Moreover, bioassays, especially in the past, frequently had the disadvantage of lacking specificity for a single CK. This problem can be largely overcome by using CK sensitive target cell lines as indicators in combination with mAbs directed against the CKs (44).

Based on the fact of the existence of both advantages and disadvantages in each method described above, the safest approach to adopt in the study of CK gene expression is to use a combination of methods in order to achieve more reliable detection results.

(iv) The current study of T cell-derived CK gene expression

According to studies by Mosmann and colleagues, as well as other groups, on long-term culture of cloned T cells, at least two distinct subsets of T helper cells have been found <u>in vitro</u> in the mouse. Named Th1 and Th2, they are differentiated based on their helper function to B cells in Ab production and the different profiles of CKs produced by each subset (9, 18). Th1 cells secrete IL-2, IFNr, granulocyte/macrophage colony stimulating factor (GM-CSF), IL-3, TNF and lymphotoxin; Th2 cells secrete IL-4, IL-5,

IL-6, IL-10, GM-CSF and IL-3. These T cell-derived CKs have been demonstrated to play an important role in isotype selection of Abs in an immune response (36, 53).

Some studies on the expression and regulation of these CK genes have been carried out up to now. Dallman et al. have studied the expression of IFNr, IL-2, IL-3, IL-4 and some other CK genes in mouse cardiac allografts by using Northern blotting and PCR techniques. They found that IFNr gene expression peaked around day 5 after transplantation based on results obtained with Northern blotting. By PCR analysis, they found that different groups of normal, syngeneic and allogeneic grafts exhibited different expression patterns of CK genes (4).

Another group, Gauchat et al., have compared IL-2, IFNr and GM-CSF mRNA expression levels in human peripheral blood mononuclear cells (PBMNC) stimulated polyclonally with mitogen PMA and anti-CD3 mAb (BMA030), by using dot blot and in situ hybridization. Their results indicated that following different stimulation procedures, different CK mRNA production profiles could be induced. They hypothesized that it could be due to the cellular heterogeneity of the PBMNC and some other unknown reasons. They have found differences in the anti-CD3 effect on IFNr and IL-2 mRNA levels. Anti-CD3 induced easily detectable IFNr and GM-CSF mRNA, but IL-2 mRNA was un-

detectable (44). Similar results were observed in IL-2 protein production in anti-CD3 in vivo stimulated C57BL/6 naive mice in the present study. IL-4 and IFNr protein was easily detected in the corresponding bioassays but IL-2 was not the case (see Results). Other reports on anti-CD3 inducing low IL-2 synthesis and mRNA expression in humans have been published (25, 54, 55). With allergen stimulation, the mRNA level studies of IL-4, IFNr and IL-2 in PBMNC showed that there was no differences in mRNA expression of these three CKs in both normal and allergic donors. IL-4 mRNA was not increased in allergic donors (44).

Regulation of early activation genes in T cells

The presently known mechanisms of T cell regulation of early activation genes (IL-4, IFNr and IL-2 included) involve two levels of controls: transcriptional and post-transcriptional. Transcriptional control has received more attention in research since it is the most effective way of controlling gene expression. Some cis-acting regulatory sequences have been identified in the 5′ flanking sequences of IL-2, IL-2R, IFNr and GM-CSF genes (40, 56-59). A series of trans-acting regulatory proteins interacting with the 5′ flanking sequences have been found : NF-AT (nuclear factor of activated T cells), NF-IL-2-A/Oct-1, NF-kB, (AP-1/Jun)/Fos and AP-3 (40, 60-66). Cyclosporin A and glucocorticoids have been shown to exert some in-

hibitory effects at the transcriptional level of control (67-69). Altogether, these regulatory DNA sequences and protein factors control the rate of transcription, the initiation and termination of the transcription of T cell activation genes.

The post-transcriptional controls include mRNA stability control and some transcriptional blocks occurring in the intervening sequences of certain genes activated after stimulation of T lymphocytes (40). Variations of the AUUA sequence found in the 3′ nontranslated region of several CK genes have been found to have a pronounced destabilizing effect on mRNA when inserted into β-globin transcriptional units (42). The transcriptional blocks have been found in IL-2 and c-myc genes (70, 71). Stimulation of cells with mitogens such as tumor promotor PMA appears to release this block and give rise to normal transcripts. The biochemical mechanism of this level of control is unclear (40).

In addition to transcriptional and post-transcriptional control of CK gene expression, other controls at translational and post translational levels, such as control of translation speed, half life of the translated molecule or modification of protein activity, are likely to be discovered as more work is done in this area (40). A summary diagram of the nuclear events occurring in T cell activation is shown in Fig. III (40).

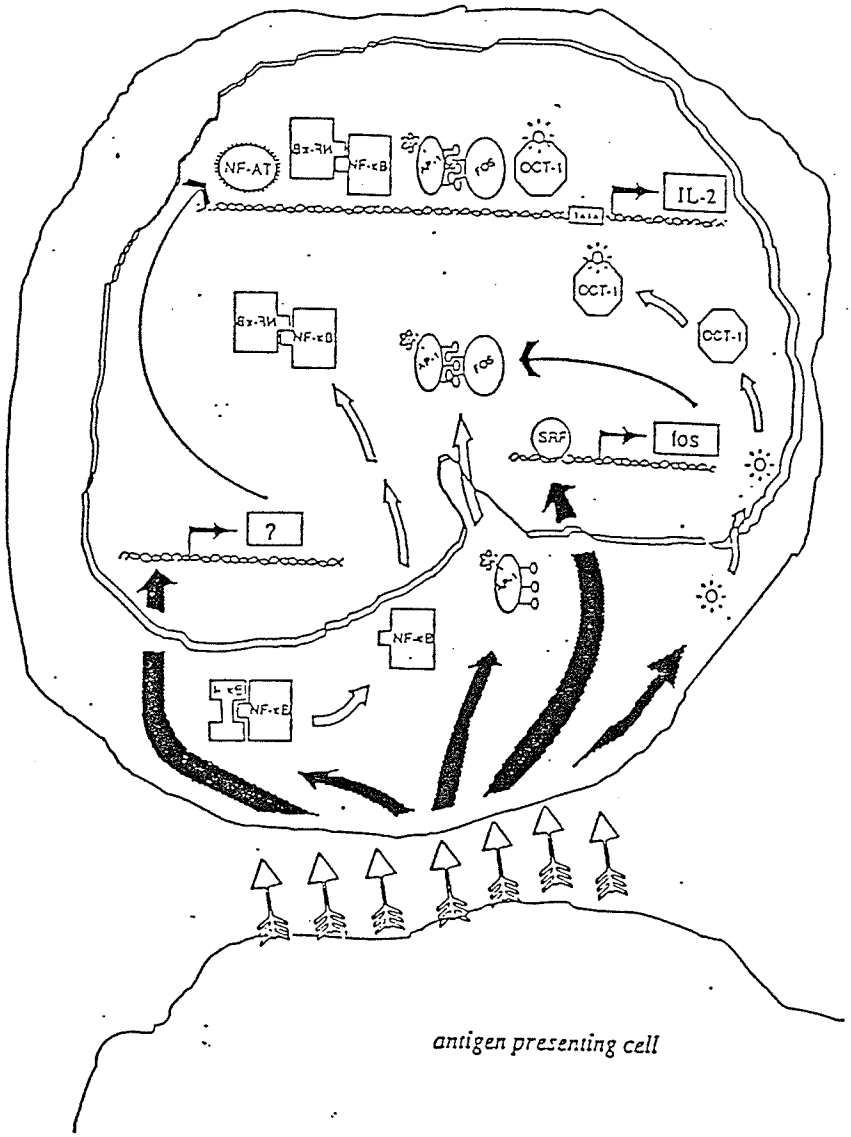


Figure III A summary of current knowledge and speculation of the nuclear events essential for T cell activation. The flow of information goes from the bottom upward toward the IL-2 gene. The enhancer of the IL-2 gene is illustrated, with the transcription initiation site indicated by an arrow. The nuclear membrane is shown as a double membrane.

CKs and allergy

(i) Allergic immune responses

Allergic immune responses (Type I Hypersensitivity) in the atopic individual are characterized by the induction of allergen-specific IgE production during the primary response and release of a series of pharmacological medators from mast cells and basophils upon second contact with the allergen, causing an inflammatory reaction and the activation of polymorphonuclear granulocytes (72). Mast cells or basophils in the skin or airways become sensitized when specific IgE molecules, produced in the primary immune response to an allergen, are bound to the high affinity Fcϵ receptors (FcϵRI) on the cell surface. Upon secondary exposure to the same allergen, a biphasic response occurs. An immediate or early response occurring within minutes is induced by the cross-linking of specific IgE molecules bound on FcϵRI on mast cells and basophils and causes granule-associated primary mediator release. A subsequent secondary or late phase response (LPR), which is largely inflammatory, occurs between 4-48 hr of the allergen exposure and is characterized by the infiltration of eosinophils, neutrophils, macrophages, lymphocytes and basophils into the reaction site, which subsequently release secondary mediators (72-74).

It has been well established that the induction and control of IgE synthesis in the primary immune response is T cell dependent (72, 75). The activated T cells are also involved in allergic inflammatory reactions through CK release to regulate the differentiation, activation and expansion of granulocytes (76).

(ii) The role of CKs in allergic immune response

To a great extent, T cells play their roles in the early and late phases of the allergic reaction through T cell-derived CKs. Studies in both mouse and human systems have shown that IL-4 is required for IgE synthesis by Ag-activated B cells (53). The mechanism involves signaling the B cell to switch from IgM synthesis to IgG_1 and IgE synthesis in the mouse, or to IgG_4 and IgE synthesis in the human, according to several reports published in the literature (77, 86-90). In the human system, experiments have shown that rIL-4 added to the peripheral blood leukocytes of atopic and normal donors could induce an increase in IgE secretion and activated B cell proliferation (72). In the mouse, anti-IL-4 Ab could block an on-going IgE response <u>in vivo</u> (53). The function of IL-4 as a switch factor in IgE production is well established now (72).

The roles of other T cell-derived CKs in the IgE production have been studied recently. IL-6 may enhance IgE synthesis in an

isotype-nonspecific fashion (78). IL-5 was shown to increase IL-4-dependent IgE production in humans (79). Low molecular weight B cell growth factor (LMW-BCGF) and IL-2 may also upregulate IgE production in the human (80, 95). In contrast, IFNr or IFNα were demonstrated to be able to decrease IgE production in vitro (72). IL-4 could downregulate IFNr production by T cells (81). Therefore, IL-4 and IFNr appear to have opposing effects in the cascade events of IgE production, and net IgE response may be determined by the balance between the production of the two CKs.

The newly described cytokine synthesis inhibitory factor (CSIF, IL-10) has been shown to have a negative effect on IFNr production in the mouse and therefore have indirect upregulating effect on IgE production (32, 33). Moreover, GM-CSF, IL-3 and IL-4 have been shown to influence the growth and differentiation of mast cells (52).

These studies demonstrate that the immediate phase of the allergic response is influenced by the quality and quantity of the T cell help (in the form of cell-cell contact and CKs) provided to the allergen-specific B cells (72).

The exact roles of T cell-derived CKs in the late phase response (LPR) have not been established, but their effects on the cell types

involved in the LPR have been studied (72). IL-3 and GM-CSF can influence eosinophil differentiation (84). IL-5 appears to be necessary for eosinophil growth and differentiation (85). IL-4 may also promote eosinophil growth in vivo in addition to its effect on other granulocytes. IL-8 can serve to activate neutrophils and lead to their degranulation (82, 83). IL-8 has also been shown to increase histamine release from basophils in the presence of IL-3 (51). In conclusion, T cell-derived CKs may control the extent of the LPR by their effects on the various cell types responsible for the LPR, although this is still an area about which much remains to be learned.

Current views of IL-4 secreting T cell existence in vivo

IL-4 (or B-cell stimulatory factor-1, BSF-1) was initially identified in the tissue culture supernatants of the thymoma line EL-4 stimulated with PMA (96). It can also be produced by many long-term T-cell lines (e.g. Th 2 clones) when stimulated with Ag or mitogens (97). A broad range of biologic activities of IL-4 have been demonstrated through in vitro studies, including a co-stimulating effect with anti-IgM Abs on resting B cells to enter S-phase (96); increasing class-II MHC molecule expression on resting B cells (98,99); increasing IgG_1 and IgE production by B cells stimulated with lipopolysaccharide (LPS, 100-103); and stimulatory effects on many

other cell types in addition to B cells : T cells and lines , mast cell lines and other hematopoietic lineage cells (104-112).

However, direct evidence of IL-4 production and the biological functions of IL-4 in vivo is still insufficient. It has become a research focus in the recent years (91). The demonstration that anti-BSF-1 mAb markedly inhibits the IgE increase in helminthic parasite N. brasiliensis infected mice was the first step towards this direction. This establishes the IL-4 requirement in IgE production in vivo (97).

There have been several papers published in recent years concluding that IL-4 secreting T cells only exist in vivo as precursors, and not as mature cells, since detectable IL-4 production in vitro from fresh spleen cells requires not only priming but also cycles of restimulation of the cells with Con A, anti-CD3 or Ag (23, 113-114). Cell frequency studies with limiting dilution analysis (LDA) also showed a high frequency of IL-2 secreting cells in Ag-CFA (complete Freund's adjuvant) immunized mice, but IL-4 secreting cells were undetectable by LDA without in vivo priming and in vitro restimulations of the primed cells requiring up to 15 days culture (115). With polyclonal stimulation in vitro, IL-4 was also reported to be undetectable (116,117). Altogether, these data seemed to suggest that IL-4 secreting T cells exist in vivo only as precursors,

since they require primary stimulation and several cycles of restimulation in vitro in order to produce IL-4.

Nevertheless, the results obtained from various other approaches, e.g. IgE production in vivo in response to protein Ag in appropriate adjuvants, and to polyclonal activators (e.g. goat anti-mouse IgD) (118-120) suggest the opposite to the data listed above. These results provide indirect evidence for the existence of mature cells in vivo capable of secreting IL-4 since IgE was produced very soon after immunization without a requirement for an extended restimulation cycles with Ag or polyclonal stimulation. In this study we have obtained direct evidence for the existence of mature T cells capable of producing IL-4 (and IFNr) mRNA and biologically active CK very rapidly following polyclonal activation in vivo or in vitro, without a requirement for multiple cycles of restimulation and extended periods of in vitro culture, previously believed to be necessary to drive IL-4 capable "precursors" to mature cells.

Summary of research objectives

The objectives of the present study are: (i) to study allergy-relevant murine CK (IL-4, IFNr and IL-2) gene expression induced by different in vivo and in vitro T cell activation stimulation with both anti-CD3 mAb and OA; (ii) to characterize the

regulation of expression of these CK genes under appropriate stimuli; (iii) to evaluate different detection methods used in assessing CK genes at two different levels, the mRNA and protein levels.

The rationale of the objectives is as follows. T cell activation can be initiated by specific recognition of antigenic peptides in the context of the MHC and mimicked by polyclonal stimulation with mitogens and by monoclonal antibodies directed against T cell receptors. This activation process can be expressed as CK gene expression, T cell proliferation and differentiation. Therefore, a direct relationship exists between the induction of T cell activation and T cell-derived CK gene expression.

The approaches chosen for this study were to use mAb anti-CD3 (145-2C11) and Ag ovalbumin as <u>in vivo</u> and <u>in vitro</u> T cell activation stimulants to naive C57BL/6 mice. IL-4, IFNr and IL-2 gene expression was examined as representative CK. The methods employed to detect CK gene expression were Northern blotting, RNA dot blotting and cell blotting for the mRNA level assessment and CK specific bioassays for the protein assessment of the expressed CK genes.

The results of this study have revealed some well characterized patterns and regulatory features of IL-4, IFNr and IL-2 gene expression

under anti-CD3 stimulation. These results also strongly suggest that IL-4 secreting T cells exist *in vivo* as mature cells, and not only as precursors.

MATERIALS AND METHODS

Mouse strains

C57BL/6 inbred mice (6-12 weeks old), bred at the University of Manitoba, were used in the present study.

Plasmids

Plasmid pcD-1 containing IL-4, IFNr or IL-2 cDNA inserts were obtained from the laboratory of Dr. T. Mosmann (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, California). E. coli strain DH5'α was used for pcD-1 transformation (see later).

Stimulants

(i) mAb:

145-2C11: Anti-CD3 mAb, initially derived by Dr. J. Bluestone (Ben May Inst., University of Chicago), was a kind gift from Dr. E. Rector's laboratory (Dept. of Immunology, University of Manitoba). Tissue culture supernatant produced in "serum-free" Nutridoma medium (Boeringer, Mannheim, Montreal, PQ) was provided.

11B11: Anti-IL-4 mAb secreted from hybridoma line 11B11 (rat IgG_1) was purified from tissue culture supernatant by ion exchange and gel filtration chromatography using the Pharmacia Biopilot system (Pharmacia Fine Chemicals, Piscataway, NJ).

XMG1.2: A rat IgG_1 anti-IFNr secreting hybridoma (9) was derived by Dr. T. Mosmann (DNAX, California). Antibody was purified from tissue culture supernatant as described above.

7D4: Anti-IL-2 receptor (R) mAb produced by hybridoma 7D4 (The American Type Culture Collection, ATCC, Rockville, Maryland) was prepared by growing ascites by injecting 7D4 cells (9 x 10^6/mouse) into irradiated (500 Rads) Balb/c recipients, i.p. and collecting the peritoneal fluid 10-14 days later.

(ii) Antigens (Ag):

Ovalbumin (OA): Chicken ovalbumin (ICN, Mississauga, Ontario) was used as Ag for <u>in vivo</u> and <u>in vitro</u> antigen-specific stimulation.

Glutaraldehyde-polymerized-ovalbumin (OA-POL): glutaraldehyde modified ovalbumin of Mr 3.5 x 10^7 was previously demonstrated to induce Ag and isotype specific inhibition of murine IgE responses to OA (26-31). OA-POL was also used as an Ag stimulant in the study.

(iii) Mitogens:

Concanavalin A (Con A) and phorbol myristate acetate (PMA) were used as polyclonal stimuli in developing the methodology for this study.

Cell lines

(i) Spleen cells:

Most of the study was carried out with freshly obtained spleen cells of C57BL/6 mice, for both RNA extraction and bioassay systems.

(ii) D10.G4.1:

A mouse helper T cell clone, which is I-A^k restricted and conalbumin specific (11), was purchased from the ATCC.

(iii) EL-4:

A C57BL/6 T cell lymphoma line that produces IL-2, as well as other cytokines, under the stimulation of mitogen PMA was obtained through the ATCC.

(iv) CT.4S:

A CTLL derived, IL-4 responsive cell line, obtained from Dr. W. Paul (National Institutes of Health, NIH) was used as an indicator cell for IL-4 specific bioassays in the presence and absence of anti-IL-4 mAb 11B11. CT.4S is highly sensitive to IL-4, responding to minimum 2-4 units (U) / ml but exhibiting with low sensitivity to IL-2 unless present at concentrations > 100 U / ml.

(v) WEHI 279:

A murine B lymphoma line (11) expressing surface u, k, was obtained from Dr. M. Hagen (University of Iowa, IA.) and used as an indicator cell line for IFNr bioassays in the presence and absence of anti-IFNr mAb XMG 1.2.. The presence of IFNr in the culture medium inhibits the proliferation of these cells in a dose dependent manner.

(vi) HT-2:

This mouse T helper clone (7), obtained from Dr. K. Rock, (Harvard Medical School, Boston, MA), was used as indicator cells for IL-2 specific bioassays, in the presence and absence of the anti-IL-2R mAb 7D4.

(vii) CTLL-2:

A monoclonal T-cell line (8) obtained from Dr. D. Rayner (University of Manitoba, MB) was dependent on exogenous IL-2 for growth and was also used as an indicator for IL-2 specific assays in the presence and absence of anti-IL-2R mAb.

CK standards (STD)

(i) rIL-4 was obtained from Dr. R. Tepper (Dana Farber Cancer Institute, Boston, MA) and used for STD proliferation curves in IL-4 bioassays at a concentration range of 250 U / ml to 7 U / ml (stock concentration: 1000 U / ml) as well as to maintain CT.4S in culture.

(ii) The STD MuIFNr, obtained from NIH, was assigned to have a potency of 1,000 International Units (IU, 3.0 log IU) following calibration with MuIFNr STD Gg02-901-533 in NIH.

(iii) Mouse (Balb/c spleen cells) Con A supernatant (mCAS) was prepared and tested for IFNr activity to be equivalent to 120 U / ml of the STD MuIFNr NIH, by Dr. X. Yang in the lab. mCAS was used in IFNr assays for STD inhibition curves at 30 U / ml (25 % dilution of original supernatant) to 0.7 U / ml of concentration range.

(iv) Rat con A supernatant (RCAS), with a half maximum proliferation concentration at 1 % in the IL-2 titration assay, or PMA stimulated

EL-4 supernatant, half maximum proliferation at 1.25 %, was used for STD proliferation curves in IL-2 bioassays at 25 to 0.7 % dilution of the original supernatant.

Strategies for stimulation of CK gene expression

Polyclonal (145-2C11 anti-CD3 mAb) and antigen-specific (OA and OA-POL) stimulation strategies employed in this study are illustrated as follows:

(i) In vivo stimulation with anti-CD3 mAb:

A single dose of 30 ug anti-CD3 Ab in serum free medium was injected i.v. into naive C57BL/6 mice. 30 minutes to 6 hours later, the mice were killed and spleens were removed. For the purpose of RNA analysis, the whole spleen was immediately frozen in dry ice for subsequent RNA extraction procedures. For cell culture, a single cell suspension of spleen cells was made in RPMI medium (Flow Laboratories, Mississauga, Ontario) with 10 % fetal calf serum (FCS, GIBCO, Burlington, Ontario), 1 % antibiotics (penicillin / streptomycin / fungizone, GIBCO), 1 % L-glutamine (Flow Laboratories) and 5 x 10^{-5} M β_2-mercaptoethanol (2 Me, Kodak, Rochester, New York). The cells were cultured at a concentration of 10 x 10^6 to 20 x 10^6 cells/ml, at 37°C, in 24-well plates (Corning, N.Y.) (2 ml /

well) for 12 to 24 hr without further stimulation. The culture supernatants were tested in bioassays for biologically active IL-4, IFNr and IL-2.

(ii) In vitro stimulation with anti-CD3 mAb:

Anti-CD3 mAb solution (30 ug/ml in serum free medium, Nutridoma, Boehringer-Mannheim) was precoated on 24-well plates, 0.3-0.5 ml/well, at 4°C, overnight, to be immobilized onto the plastic surface. Then, the wells were washed with serum free RPMI medium three times. Naive C57BL/6 mice were sacrificed and whole spleen cells were made into single cell suspensions in RPMI and cultured in anti-CD3 coated 24-well plates at 10 x 10^6 cells / ml (2 ml / well) for 3 to 24 hr. The supernatant was harvested for later bioassay of CK production. The cells were spun down, cooled on ice and the pellets were frozen at -70°C for RNA extraction.

(iii) In vivo Ag-specific stimulation:

OA stimulation: Naive C57BL/6 mice were immunized with 2 ug OA absorbed to 2 mg alum ($Al(OH)_3$) per mouse, by i.p. injection (0.5 ml / mouse). 4 days after immnization, the mice were killed and spleens were taken out to prepare single cell suspension in 10 % RPMI medium (as before). The cells were cultured at 8 x 10^6 cells /

ml with a secondary OA (1 mg/ml) in vitro stimulation. After 12 to 18 hr culture, the supernatant was harvested to test IL-4, IFNr or IL-2 activities by bioassay.

OA-POL stimulation: 80 ug OA-POL in 0.5 ml NaCl was injected into each naive mouse, i.p. on days -16, -14 and -12. On day 0, the mice were challenged with 2 ug OA-alum (0.5ml / mouse) i.p.. The mice were then killed on different days (from day 3 to 7); spleen cells were made into single cell suspensions and T cell-enriched by panning on sheep-anti-mouse Ig (obtained from Dr. A. Sehon's laboratory, Dept. of Immunology, UM) coated petri dishes. The enriched T cells were harvested and used directly in cell blotting for RNA study without further in vitro stimulation.

(iv) Mitogen stimulation:

Con A (5 ug/ml) was used to stimulate C57BL/6 spleen cells and T cell clone D10.G4.1 cells in vitro to induce T cell activation and LK production. PMA was used to stimulate EL-4 line (10 ng / ml) in vitro for IL-2 production.

RNA

(i) Sources:

Total cellular RNA was extracted from C57BL/6 spleen cells, spleen T cells, EL-4 thymoma cells and D10.G4.1 T helper clone cells.

(ii) Isolation of total cellular RNA:

Guanidinium-isothiocyanate / Cesium chloride (CsCl) method (10) was used to prepare most of the RNA samples in the study.

The spleen was treated as a tissue under the guidance of Dr. L. Murphy (Dept. of Biochemistry, University of Manitoba) as follows: the mice were sacrificed at the times indicated (at Results) following polyclonal or antigenic stimulation. Whole spleens were taken out quickly and frozen in dry ice to minimize RNase activity. Then each spleen was put in 9 ml 4 M guanidinium isothiocyanate (GIBCO, BRL) buffer (nuclease inactivating solution) and homogenized by a Brinkmann homogenizer for 2 to 3 sec at high speed for 2 to 3 times. The homogenized spleen was placed on the top of a 4 ml 5.7 M CsCl (BRL) / 0.1 M EDTA (pH 7.5 Mallinckrodt, Canlab, Winnipeg, MB) cushion in a Quick-Seal Ultra-Clear™ centrifuge tube (5/8 x 3 in. Beckman, Palo Alto, CA). The top was sealed. Ultracentrifugation was performed with a 70.1 Ti fixed angle rotor in a Beckman L-70 ultracentrifuge at 25,000 rpm, 22°C for 22 hr.

For tissue culture cells (EL-4, D10.G4.1), confluent cultured cells

were spun down and pellets were frozen in -70°C. Upon RNA extraction, 4 M guanidinium buffer was added to the pellet during its thawing (100-150 x 10^6 cells / 9 ml buffer). The cells were lysed and DNA was sheared by using a 20-gauge needle fitted syringe. The cell lysate was placed on top of a 4 ml CsCl cushion and ultracentrifuged under the same conditions as the spleen samples.

After ultracentrifugation, each RNA pellet was dissolved in 1 ml TE (10 mM Tris.Cl, 1mM EDTA, pH 7.6), and precipitated with final 0.3 M sodium acetate (NaOAc, pH 5.5) and two fold volume of absolute ethanol (ETOH, 100%), at -20°C, overnight. Final RNA preparation was recovered by recentrifuging in a SS34 fixed angle rotor at 16,000 rpm, 4°C, for 1 hr and redissolving the RNA pellet in TE (pH 7.6) buffer. The concentration of the RNA preparation was determined by spectrophotometric absorbance at 260 nm with a ZEISS PMQ3 spectrophotometer : an optical density (OD) of 1 was taken to correspond to 40 ug / ml RNA and single-stranded DNA (10). Approximate yield of total RNA of 5 x 10^6 cells should be ~ 50 ug (10^{-5} ug total RNA / mammalian cell) (10). The isolated total RNA was used in Northern or dot blots for the mRNA analysis of the CK gene expression.

A proportion of RNA samples in the study were extracted according to the guanidinium / hot phenol method (10). Briefly, cells contained

in 4 M guanidinium isothiocyanate buffer and phenol (60°C) were lysed and DNA was shared by passing the solution mixture through an 18-gauge needle syringe. Half volume of RNA extraction buffer (0.1 M sodium acetate, pH 5.2, 10 mM Tris.Cl, pH 7.4 and 1 mM EDTA) and equal volume chloroform were added to the cell lysate, and cooled on ice for 10 min. Aqueous containing RNA was recovered by centrifugation at 2000 g for 10 min at 4°C. The aqueous phase was reextracted using phenol and chloroform to purify the RNA preparation. RNA was precipitated with salt and ethanol as before and recovered by centrifugation at 12,000 g for 1 hr. The RNA pellet was dissolved in a solution containing 0.1 M Tris.Cl, pH 7.4, 50 mM NaCl, 10 mM EDTA and 0.2 % SDS, digested with proteinase K (200 ug/ml final), reextracted with phenol (60°C) and chloroform, and precipitated with salt and ethanol. The recovered RNA from centrifugation was ready for use.

Isolation of cDNA inserts

A series of experiments was carried out in order to generate cDNA inserts of IL-4, IFNr and IL-2 for the RNA hybridization analysis.

(i) Transformation of plasmids into E. coli:

The calcium chloride procedure (16) was used to transform pcD

plasmid vectors containing IL-4, IFNr or IL-2 cDNA into the bacterium E.coli strain DH5'α (BRL). 1 to 2 DH5'α colonies were picked from the stock and spread onto a LB (Luria-Bertani)-agar (Difco laboratories, Detroit, Michigan) plate (no ampicillin). The plate was incubated at 37°C overnight. Freshly grown colonies were dispersed into LB growth medium and incubated at 37°C overnight with agitation. Competent cells were prepared by transfering 5-10 ul actively growing E. coli suspension into 10 ml LB with 10 mM $MgCl_2$ medium, to grow to ~5 x 10^7 cells / ml (log phase of growth). The cells were spun down and treated with 100 mM $MgCl_2$ and 75 mM $CaCl_2$ to become competent for transformation (16). The plasmid (100 ng / ul) was added to the competent cells (1 ul plasmid into 100 ul competent cells) and transformed into E.coli cells by the heat shock procedure. The transformed E.coli cells were selected by growing in ampicillin (50-100 ug / ml, Sigma, St. Louis, MD) containing LB-agar medium.

The selected E.coli colonies were picked out and seeded into LB growth medium with 50-100 ug / ml ampicillin and incubated at 37°C, with agitation overnight for bacterial growth and plasmid expansion. The fully grown culture was spun down and the cell pellet was collected for plasmid DNA extraction.

(ii) Plasmid DNA extraction:

The pcD plasmid DNA was isolated from E.coli cells by rapid alkaline extraction of plasmid DNA method (12). The cells were lysed in a freshly made solution containing 125 ul of 10 % SDS and 40 ul of 0.5 N NaOH per ml. The bacterial proteins were precipitated by final 2.5 M ammonium acetate (NH_4OAC). The plasmid DNA and bacterial proteins were separated by centrifugation in a SS34 rotor at 15,000 rpm, for 1 hr at 4°C. The supernatant which contained the plasmid DNA was saved and the precipitates were discarded. The DNA was then precipitated by 2.5 volume of ETOH at -20°C or equal volume of isopropanol at 4°C. The DNA pellet was recovered by centrifugation. To increase the purity of the isolated DNA, a second lysis procedure and RNase A (1-10 ug / ml final) and proteinase K (1-10 ug / ml) digestions of the sample were required. The final DNA precipitate pellet was washed, dried and redisolved in TE buffer (pH 7.6). DNA concentration was spectrophotometrically determined : at wave length 260 nm, with an OD of 1 corresponding to 50 ug / ml double-stranded DNA.

(iii) RE digestion of cDNAs:

After obtaining the plasmid DNA from E.coli cells, various restriction endonucleases (RE) were used to generate corresponding CK cDNA inserts from the plasmids. An approximately 800-base-pair (bp) fragment containing the full-length IL-4 cDNA insert (585 bp, ex-

cluding poly (A) tail) (13) was obtained between the two RE Xho I (Pharmacia, Baie D'Urfe, Quebec) sites in the vector. The IFNr cDNA fragment was generated by Bam HI (Pharmacia) digestion giving rise to a 1.4 kilobase (kb) long fragment containing the full-length IFNr cDNA insert (17). The IL-2 fragment was digested with REs Pst I (Pharmacia) and Acc I (BRL). Since there were 3 Pst I sites in the vector and 4 different Acc I sites at the 3' end of the IL-2 cDNA sequence (14), Pst I and Acc I digestion generated 2 smaller fragments of about 630 bp and 750 bp each in length. Both contain the full length single open reading frame of the IL-2 insert. Both fragments were collected.

The digested IL-4, IFNr and IL-2 inserts were separated from the rest of the plasmid DNA on 1 % agarose (BRL) gel. The inserts were collected either directly from the gel under high voltage electrophoresis viewed with an ultra-violet (UV) light (10), or by the centrifugal filtration method (15): Briefly, the exact band of the insert was cut out on the gel, the gel piece was wrapped in a Millipore (Type GV) membrane and the eluted insert was collected by centrifugation. The collected inserts from both methods was extracted once with equal volume of phenol (Lawrence Packging Supply Corp, LP) and chloroform (Mallinckrodt) and once with chloroform to purify the cDNA preparation. The insert cDNA was then precipitated with salt and ETOH. After centrifugation, the pellet was washed,

dried and redissolved in TE (pH 7.6) buffer. The isolated IL-4, IFNr and IL-2 inserts were used as cDNA probes for RNA hybridization studies.

Hybridization techniques

(i) Blotting techniques:

a. Northern blotting:

The isolated total RNA was denatured in denaturation solution and separated in 1 % denaturing agarose gel (containing formaldehyde) at 30 volts, overnight (10). The separated RNA samples were photographed under UV light. The RNA was transferred from the gel onto a Hybond-TM N membrane (Amersham, Oakville, Ontario) in the presence of 20 x SSC (sodium chloride / sodium citrate solution). A stack of blotting paper towels was placed on the top of the Hybond nylon membrane to facilitate transfer of nucleic acids from the gel to the membrane. After 24 hr transferring, the membrane was taken out and viewed under UV light. The transferred RNA was crosslinked to the Hybond membrane by UV radiation for 5 min. The crosslinked membrane was ready for prehybridization.

b. RNA dot (slot) blotting:

Using the Hybond membrane and dot blot apparatus (BRL), the membrane was treated first with sterile water and then with 20 x SSC. The membrane was put into the apparatus, and an appropriate amount of total cellular RNA (from 1 to 20 ug) was loaded into each well. The membrane was drained with a vacuum, then rinsed with 6 x SSC, and air dried. RNA was crosslinked to the membrane by UV radiation and ready for prehybridization.

c. Cell blotting:

Freshly obtained, living cells (spleen cells, tissue culture cells) were loaded onto the membrane fixed in the dot blot apparatus (15). The number of cells loaded in each well could range from 10^3 to 10^6. After drained with a vacuum, the membrane was taken out, and treated with the detergent sodium dodecyl sulfate (SDS, 0.2 % in 2 x SSC) and the denaturing reagent formaldehyde (10-20 % in 2 x SSC) to lyse the cells on the membrane and linearize RNA molecules in order to bind to the membrane (double stranded DNA would not bind to the membrane). The membrane was also treated with proteinase K (100 ug / ml) to digest the protein of the cells, which might compete with RNA molecules to bind to the membrane. The membrane was then washed with 2 x SSC and air dried. After crosslinking RNA to the membrane by UV light, the membrane was ready for prehybridization.

(ii) Nick translation:

The isolated IL-4, IFNr and IL-2 cDNA inserts were labelled with (α-^{32}P)-dCTP (ICN) employing nick translation (10) and used as probes for RNA hybridization study. Each nick translation reaction contained 1 ul of each unlabelled dATP, dTTP and dGTP (0.4 mmol / 1 stock, BRL), (α-^{32}P)-labelled dCTP (50 uCi), cDNA insert (100 ng), 1 ul DNA polymerase I and DNase I (250 u / 625 ul stock, BRL) and nick translation buffer (according to Nick Translation Kit instruction by Molecular Biology Boehringer Mannheim). The reaction was carried out at 15°C for 90 min. The reaction was stopped by the addition of 2.5 ul 0.5 M EDTA. The labelled cDNA was separated from unincorporated dNTPs on a Sephadex G-50 spun column (10). The incorporation rate of (α-^{32}P)-dCTP was > 30 % in the experiments. The specific activity of the labelled cDNA obtained in each reaction varied between 10^7 to 10^8 cpm / ug.

(iii) Hybridization:

The RNA containing membrane was prehybridized in a sealed plastic bag with prehybridization solution (5 x Denharts, 6 x SSC, 0.1 % SDS, final 10-15 ug / ml denatured herring sperm DNA (Boehringer Mannheim GmbH, W. Germany) (10) at 65°C for > 4 hr. Then the labelled cDNA probe was denatured at 100°C for 3 min and added into

the prehybridization bag to hybridize with the RNA on the membrane at 65°C for 24 hr. The membrane was taken out and washed with 2 x SSC-0.1 % SDS, 3 times, at room temperature and 65°C for 15 min each. More stringent wash of 0.2 x SSC-0.1 % SDS at 65°C was used if the membrane showed a high background counts on a geiger counter. The membrane was air dried and exposed to an X-ray film (Kodak) with intensifying screens (Dupont Cronex Lightning Plus, Picker, Wilmington, DE) at -70°C for different periods of time to get the optimal autoradiographic signal.

Bioassays

CK specific bioassays were used to assess the level of functional CK obtained. The bioassays were set up with 96-well-flat-bottom plates (Corning) in the presence of indicator cells, positive and negative controls and test supernatants. After 24 to 48 hr culture, ^{3}H-thymidine (ICN) was added into the culture (1 uCi / well) 12 to 24 hr prior to harvesting. The proliferation (IL-4, IL-2 assays) or inhibition of proliferation (IFNr assay) of the indicator cells was expressed in cpm or dpm and, in some cases, converted to units of activity as compared to reference STDs (see above).

(i) IL-4 assay:

CT.4S cells (maintained in culture with rIL-4 at 800 U/ml) were used as indicator cells for the production of biologically active IL-4. Highly viable cultures (>95 % viability) were washed three times with 10 % RPMI in order to remove the rIL-4 in the maintaining medium. CT.4S cells were added at 7,000-10,000 cells / well in each assay. rIL-4 was used as a positive control at a concentration range of 7 to 250 U / ml in different wells. 10 % RPMI medium was used as negative control of the assay. Test supernatants were from appropriately stimulated cultures. The assay was set up for 48 hr. CT.4S cells could respond to the different amounts of IL-4 in each well by proliferation in a dose dependant manner. The cells were harvested by a PHD cell harvester (Cambridge Technology Inc. Watertown, MA) onto glass fiber filter strips (Cambridge Technology Inc.) in scintillation vials (Fisher, Ottawa, Ontario). The filters were air dried, and scintillation fluid (2 ml / tube, Beckman) was added. The extent of proliferation was measured by a scintillation analyzer (Packard, Meriden, CT) according to the incorporation of ^{3}H-thymidine (24 hr after adding) into the CT.4S cells (cpm or dpm). mAb 11B11 (anti-IL-4) was used in parallel assays to confirm that the proliferation observed was specifically due to IL-4.

(ii) IFNr assay:

WEHI 279 cells were used as indicator cells. Highly viable cultures

(>95 %) were diluted to 10^5 cells / ml directly from maintaining cultures (in regular 10 % RPMI medium) without washing and added at 10,000 cells / well in the assay. The proliferation of WEHI cells was inhibited by the presence of IFNr in the culture. Mouse (Balb / c) Con A supernatant was used as STD IFNr containing supernatant (positive control) at concentrations from 0.7 to 30 U / ml in different wells. 10 % RPMI was used as negative control. Test supernatants were from stimulated cultures. After 48 hr culture, ^{3}H-thymidine was added to the assay. 20 hr later, the assay was harvested and the extent of inhibition of proliferation of the WEHI cells was measured based on the amount of ^{3}H-thymidine incorporation. mAb XMG 1.2 (anti-IFNr) was used in parallel cultures to determine if the inhibition of proliferation was due to IFNr in the culture.

(iii) IL-2 assay:

The indicator cells for IL-2 assay were HT-2 (or CTLL-2) cells which would respond to exogenous IL-2 by proliferation in a dose dependant manner. The cells were maintained in IL-2 containing medium (10 % FCS, 10-20 % RCAS, 1 % L-glutamine, 1 % penicillin / streptomycin / fungizone, 1 % nonessential amino acid (NEAA, Flow Laboratories), 1 % sodium pyruvate (Flow Laboratories), 5 x 10^{-5} M 2Me). Cells (viability > 95 %) were washed three times with 10 % RPMI medium with-

out IL-2 and added into each assay at 10,000 cells / well. Titrated RCA supernatant was used as STD IL-2 containing supernatant (positive control) and 10 % RPMI medium was used as negative control in each assay. Test supernatants were from stimulated cultured cells. After 24 hr culture, ^{3}H-thymidine was added into each well of the assay. 10-12 hr later, the wells were harvested, scintillation fluid was added and proliferation of the cells was measured on the scintillation analyzer. mAb 7D4 was used to test the specificity of HT-2 and CTLL-2 responses to IL-2.

RESULTS

Establishment of methodology

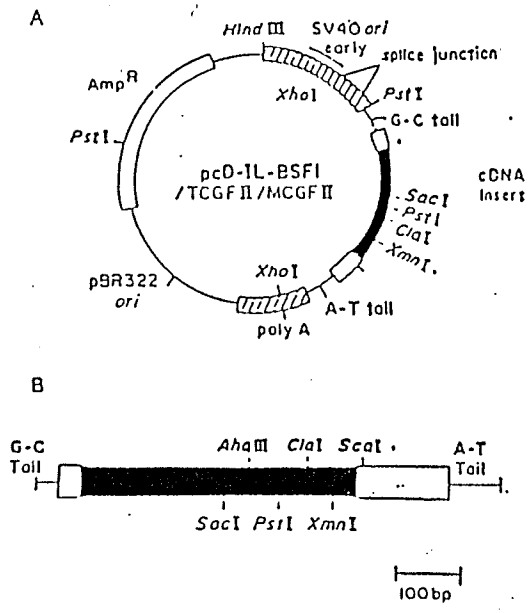
During the establishment of the methodology of various RNA blotting and hybridization techniques and CK specific bioassays for this study, initial experiments were carried out with mitogens (Con A, PMA), since they are strong polyclonal stimulators. mRNA synthesis for IL-4, IFNr and IL-2 following *in vivo* and *in vitro* stimulation of spleen cells from naive mice were assessed by hybridization using Northern, cell and RNA dot blots with CK-specific cDNA probes. The relationship between CK mRNA synthesis and the production of active CK protein was assessed with bioassays as described above.

To conduct RNA blotting analyses, the first step is to obtain a clean and specific preparation of the CK cDNA probe. The plasmid construct of each pcD vector containing IL-4, IFNr or IL-2 cDNA insert is shown in Figure 1. These plasmid vectors were transformed into E. coli. The expanded plasmid DNA was extracted by a rapid alkaline extraction method. The cDNA insert of each CK was then obtained by digestion with appropriate REs, and isolated for use as CK specific probes in the RNA blot hybridizations (for details of above procedures, see Materials and Methods). The results of RE digestion of the IL-4, IFNr and IL-2 plasmids are shown in Table 1. Each

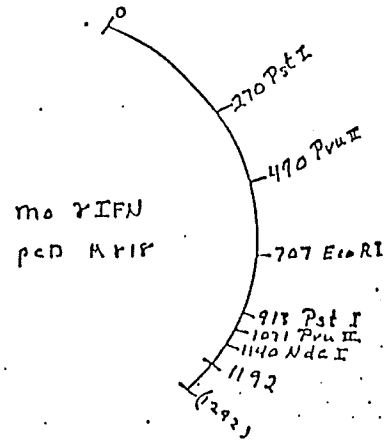
Fig. 1: Cloning vectors of IL-4, IFNr and IL-2

1A, 1B and 1C displayed represent the construction of pcD-IL-4, pcD-IFNr insert and pcD-IL-2 respectively. RE sites on the inserts are shown in each Figure.

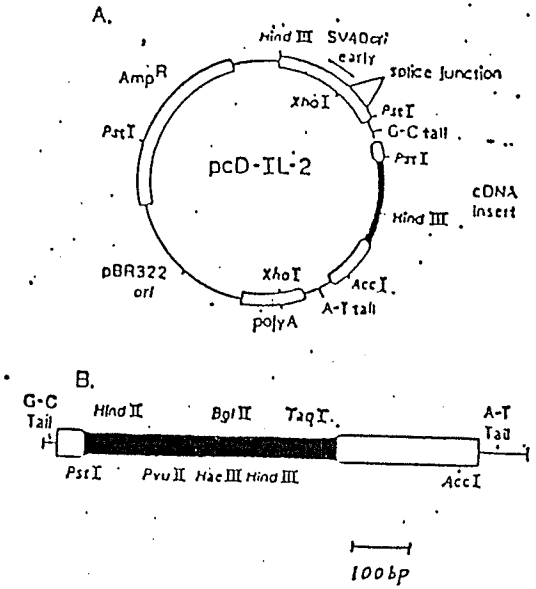
Fig. 1


A
HindIII
SV40 ori
early
splice junction
AmpR
XhoI
PstI
G-C tail
pcD-IL-BSF1
/TCGF II /MCGF II
cDNA
Insert
SacI
PstI
ClaI
XmnI.
pBR322
ori
XhoI
A-T tail
poly A
B
G-C
Tail
AhaIII
ClaI
ScaI
A-T
Tail
SacI
PstI
XmnI
100bp

1A


0
270 PstI
490 PvuII
mo γIFN
pcD Krif
707 EcoRI
917 PstI
1071 PvuII
1140 NdeI
1193
(6293)

1B


A.
HindIII
SV40ori
early
splice junction
AmpR
XhoI
PstI
G-C tail
pcD-IL-2
PstI
cDNA
Insert
HindIII
pBR322
ori
XhoI
AccI
A-T tail
polyA
B.
G-C
Tail
HindII
BglII
TaqI.
A-T
Tail
PstI
PvuII
HaeIII
HindIII
AccI
100bp

1C

Table 1: Restriction endonuclease digestion of IL-4, IFNr and IL-2 inserts

The size of each cDNA insert, the REs used in the digestion and the sizes of the digested fragments, each containing a full-length cDNA insert, are shown.

Table 1

LK	Size of cDNA insert (bp)	RE used	Size of fragment generated (bp)
IL-4	585	Xho I	800
IFNr	1292	Bam HI	1,400
IL-2	825	Pst I Acc I	630, 750

fragment contains a full-length cDNA insert of the correlate CK. The gel picture and the purity of each isolated CK insert is shown in Fig. 2. The picture shows preparations of the isolated IL-4, IFNr and IL-2 inserts free of any other size DNA fragments and RNA contamination (2B lanes 1, 2 and 4), which is important for specific RNA signal detection in the RNA blot hybridization. After obtaining pure and clean CK cDNA inserts, various methods of detection and quantitating CK mRNA were evaluated.

(i) Northern blot analysis of CK mRNA synthesis:

For a Northern blot, a good quality of total RNA preparation is required. Experiments were carried out with polyclonally activated, heterogeneous spleen cell populations. The sample RNA preparation from anti-CD3 stimulated naive mouse spleen cells is displayed in the gel picture shown in Fig. 3A. The 28 S and 18 S rRNA (ribosomal RNA) bands and the spread mRNA can be seen. The bottom part of the sample is tRNA (transfer RNA). The RNA sample was used in the Northern blotting test and hybridized to an IL-2 cDNA probe, the result of which is shown in Fig. 3B. The 900 bp IL-2 mRNA species was detected.

(ii) Cell blot analysis of CK mRNA synthesis:

Establishment of a direct and sensitive cell blot test for CK mRNA

Fig. 2: RE digested and isolated IL-4, IFNr and IL-2 inserts

1 % mini agarose gel electrophoresis of plasmids and inserts.

A: Xho I digested IL-4 plasmid is shown in lanes 2, 3 and 4 (3 ug/lane). The undigested pcD-IL-4 is shown in lane 1. Bam HI digested IFNr plasmid is shown in lanes 6, 7 and 8. Lane 5 shows intact pcD-IFNr plasmid.

B: The isolated IL-4 and IFNr inserts are shown in lanes 1 and 2. pcD-IL-2 and isolated IL-2 insert (doublet) are shown in lanes 3 and 4.

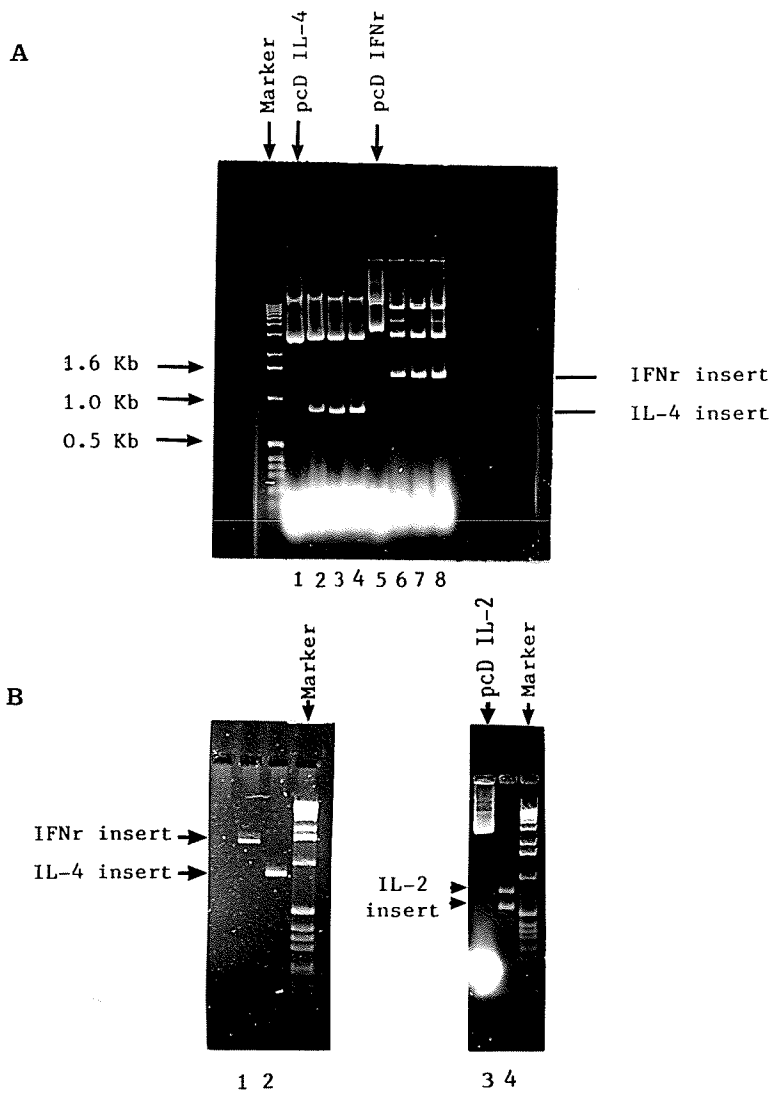

A
Marker
pcD IL-4
pcD IFNr
1.6 Kb
1.0 Kb
0.5 Kb
IFNr insert
IL-4 insert
1 2 3 4 5 6 7 8
B
Marker
pcD IL-2
Marker
IFNr insert
IL-4 insert
IL-2
insert
1 2
3 4

Fig.2

Fig. 3: Total RNA sample and Northern blot

A: Total RNA was extracted from anti-CD3 mAb stimulated C57BL/6 spleen cells by guanidinium-isothiocyanate/CsCl method (see Materials and Methods) and separated on 1% agarose gel. The 28 S (~5 Kb) and 18 S (~2 Kb) rRNA bands are indicated. The rest of the sample is mRNA. tRNA is at the bottom.

B: Northern blot. The separated RNA was used in Northern blotting and hybridized with an IL-2 cDNA probe (see Materials and Methods). The detected 900 bp IL-2 mRNA species is indicated.

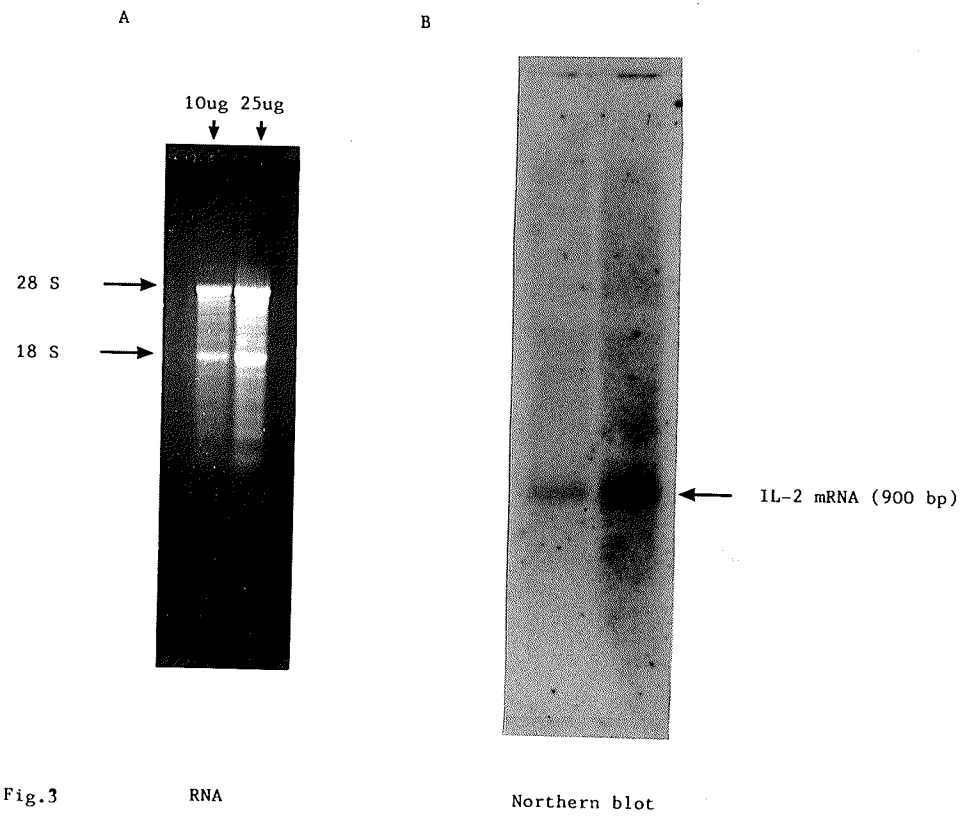


Fig.3 RNA Northern blot

assessment was carried out using Con A induced IL-4 gene expression in the T cell clone D10.G4. The blots were hybridized to IL-4 cDNA insert to evaluate IL-4 gene expression following activation of the cells by Con A. The results are shown in Fig. 4A. Different numbers of cells ranging from 2×10^3 to 2×10^5 were loaded on each spot. HT-2 cells were the negative cell control. Herring sperm DNA and plasmid pcD-IL-4 were the included as negative and positive DNA controls of the blot. As shown, positive IL-4 signal was detected at spots with 2×10^4 and 2×10^5 cells. HT-2 was barely hybridized to the IL-4 probe. The slight hybridization shown on the herring sperm DNA spots is considered to be nonspecific background noise since it is much weaker compared to the positive signal of the D10 cells and washable with high stringency washings.

(iii) RNA dot blot analysis of CK mRNA synthesis:

RNA dot blot testing was carried out using Con A stimulated naive spleen cells <u>in vitro</u>. Total RNA was extracted from the T cells, of the stimulated spleen cell population, enriched by panning on sheep anti-mouse Ig coated petridishes. The RNA was dotted on the membrane and hybridized with the IFNr insert. The result is shown in Fig. 4B. Rat-basophil-leukemia RNA (RBL, obtained from Dr. A. Froese's laboratory, Dept. of Immunology, UM), herring sperm DNA and pcD-IFNr were the controls of the blot. The positive IFNr signal could be

Fig. 4: Cell blot and RNA dot blot of IL-4 and IFNr detection

A: Cell blot. Con A (5 ug/ml) stimulated D10.G4 culture cells were used in cell blotting and hybridized with IL-4 cDNA insert probe. HT-2 cells were used as negative control. pcD-IL-4 and herring sperm DNA were used as DNA positive and negative controls.

A: RNA dot blot. C57BL/6 spleen cells were stimulated with Con A (2 ug/ml) at 5 x 10^6 cells / ml for different hours. The cells were harvested and T-enriched by panning. RNA was extracted from enriched T cells and used in dot blotting. IFNr insert was the probe. RBL RNA was the RNA negative control. Herring sperm DNA and pcD-IFNr were used as DNA negative and positive controls.

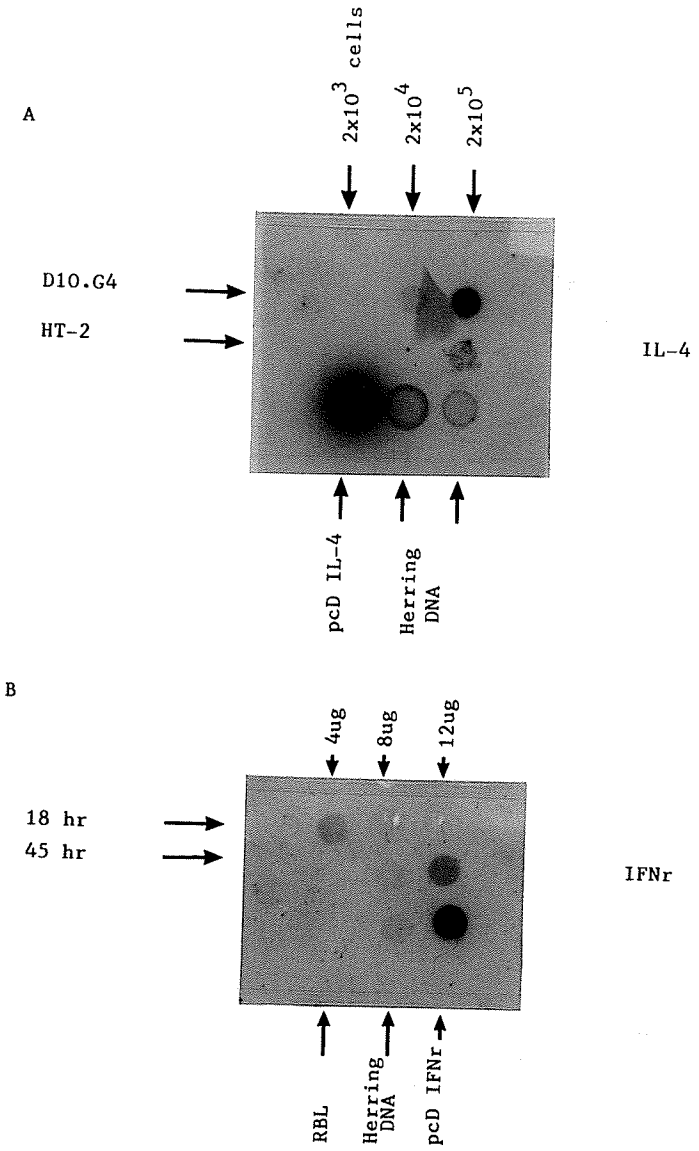

A
2x10^3 cells
2x10^4
2x10^5
D10.G4
HT-2
IL-4
pcD IL-4
Herring DNA
B
4ug
8ug
12ug
18 hr
45 hr
IFNr
RBL
Herring DNA
pcD IFNr

Fig. 4

detected 18 hr to 45 hr after Con A stimulation.

The results from the above experiments demonstrated that IL-2, IL-4 and IFNr mRNAs were detectable by each of the three independent approaches: Northern, cell and RNA dot blot analysis.

(iv) Establishment of bioassays for IL-4, IFNr and IL-2:

The sensitivity and specificity of each bioassay was examined with anti-IL-4 (11B11), anti-IFNr (XMG 1.2) and anti-IL-2R (7D4) mAbs, respectively. The details of each type of bioassay were as described in Materials and Methods. Briefly, each mAb was added into the corresponding assay to examine if the CK effect in that assay (proliferation or inhibition of proliferation) could be blocked by the Ab. The results are shown in Fig. 5, 6 and 7. They indicate that all three mAbs were able to block the correlate CK effects on indicator cells to the background level, indicating that the bioassay results obtained were specifically due to the CK being tested.

By accomplishing the above experiments, The CK specific molecular and bioassay methods were established and ready for use in the research for the project.

Fig. 5: The specificity of the CT.4S response to IL-4

The specific response of CT.4S cells to IL-4 was examined using mAb 11B11 (anti-IL-4) at 0.8 ug / well. rIL-4 was used as IL-4 source. 11B11 could block CT.4S proliferation to background levels indicating the proliferation was specifically due to IL-4.

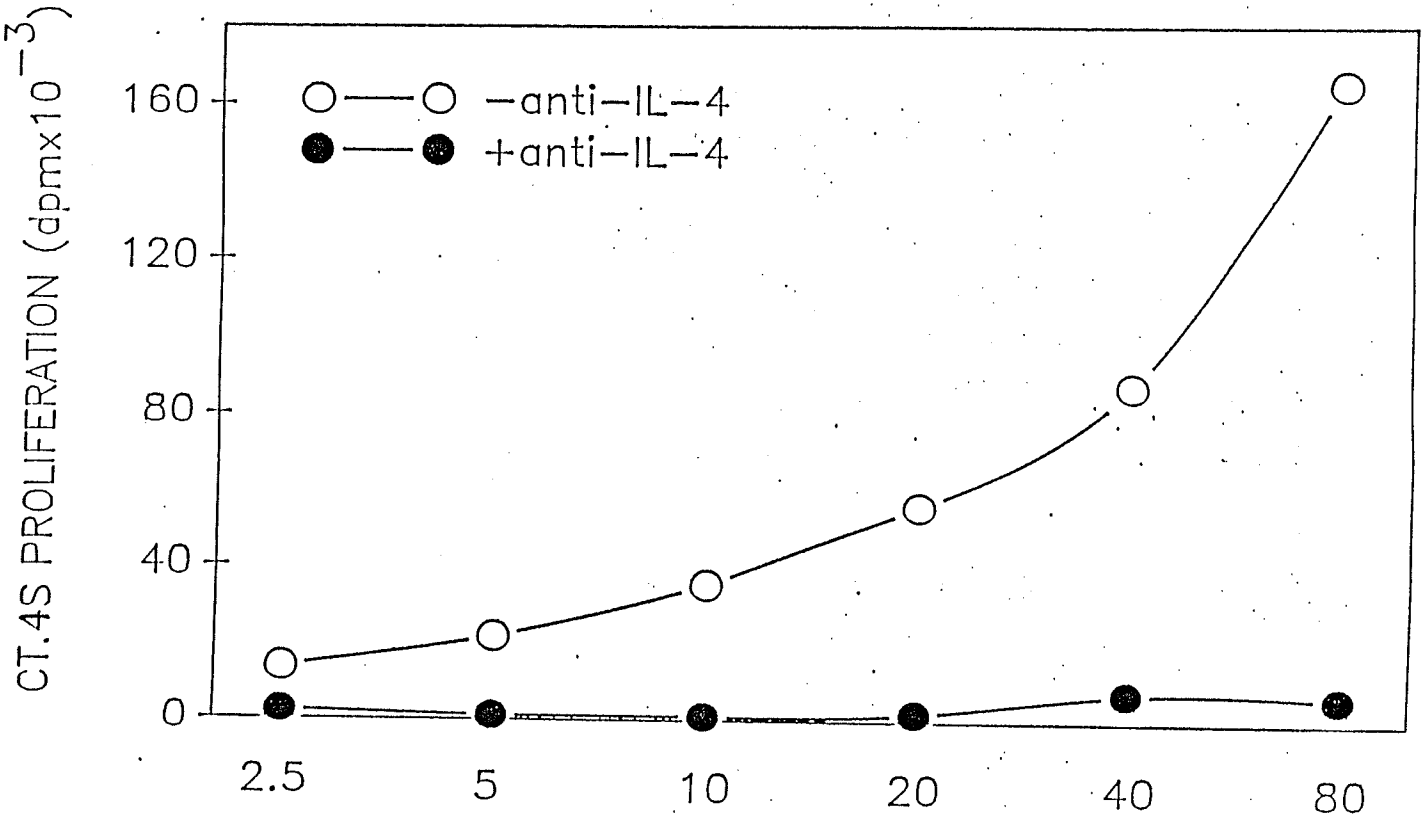

CT.4S PROLIFERATION (dpmx10^{-3})
160
120
80
40
0
O—O −anti−IL−4
●—● +anti−IL−4
2.5
5
10
20
40
80
rIL−4 (UNIT)

Fig.5

Fig. 6: The specificity of WEHI 279 response to IFNr

mAb XMG 1.2 (anti-IFNr, 150 U / ml, 25 ul / well) was used to test the specificity of WEHI 279 response to IFNr (inhibition of proliferation). mCAS (Balb / c spleen con A supernatant) was the IFNr source. XMG 1.2 could block the inhibition effect of IFNr on WEHI cell proliferation.

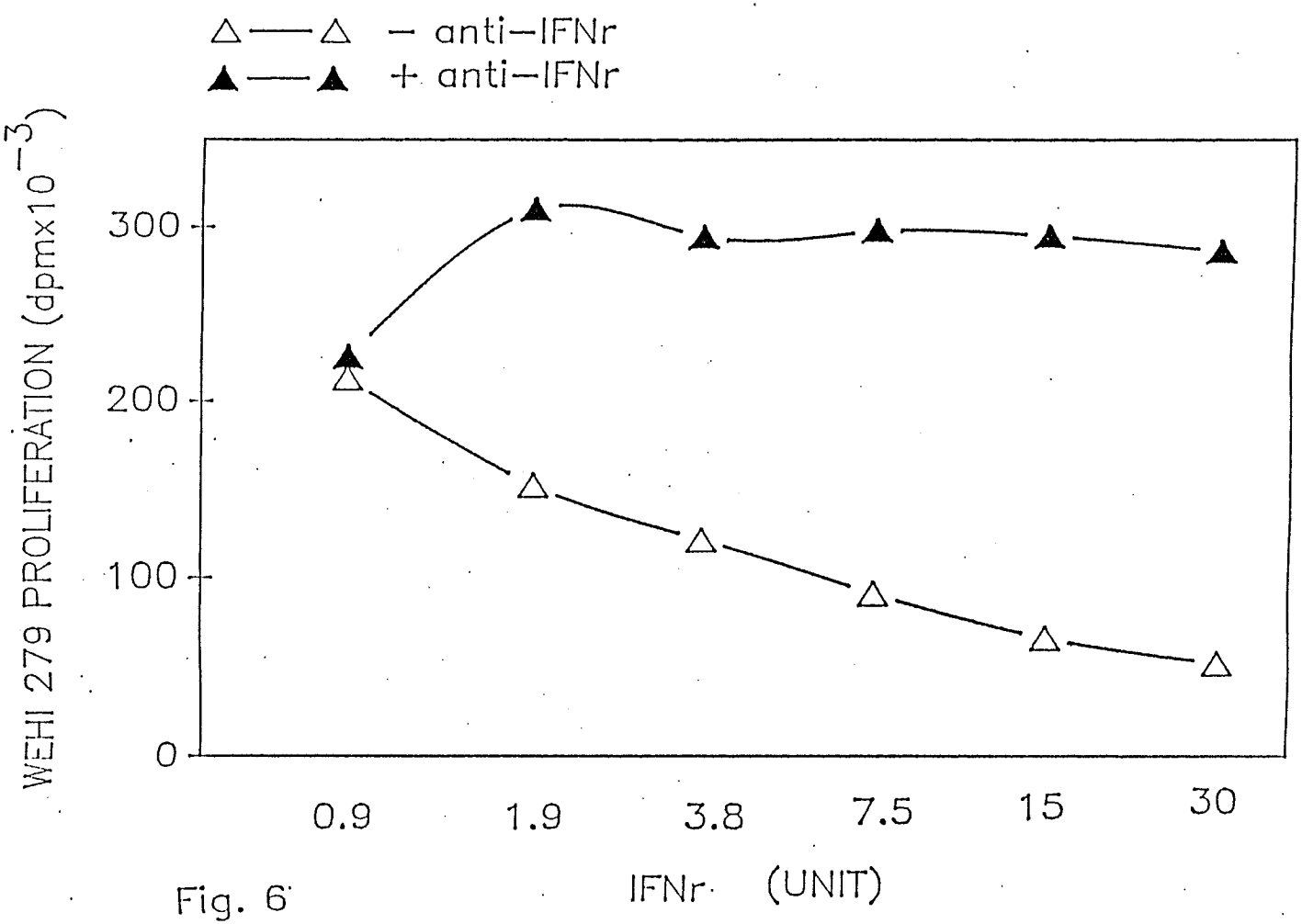
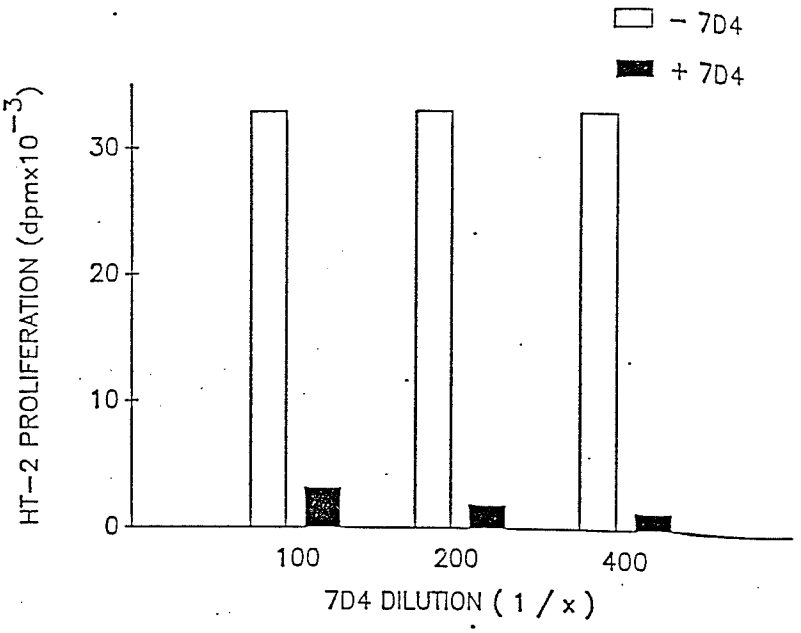

△——△ – anti–IFNr
▲——▲ + anti–IFNr
WEHI 279 PROLIFERATION (dpmx10^{-3})
300
200
100
0
0.9
1.9
3.8
7.5
15
30
IFNr (UNIT)

Fig. 6

Fig. 7: The specificity of HT-2 and CTLL-2 responses to IL-2

mAb 7D4 (anti-IL-2 receptor) prepared from ascites was used at different dilutions to assay the specificity of HT-2 (top) and CTLL-2 (bottom) responses to IL-2. RCAS (rat spleen con A supernatant) was used as IL-2 source at 0.6 % of the original supernatant. The proliferation of both HT-2 and CTLL-2 was blocked by mAb 7D4.


- 7D4
+ 7D4
HT-2 PROLIFERATION (dpmx10^{-3})
30
20
10
0
100
200
400
7D4 DILUTION (1 / x)

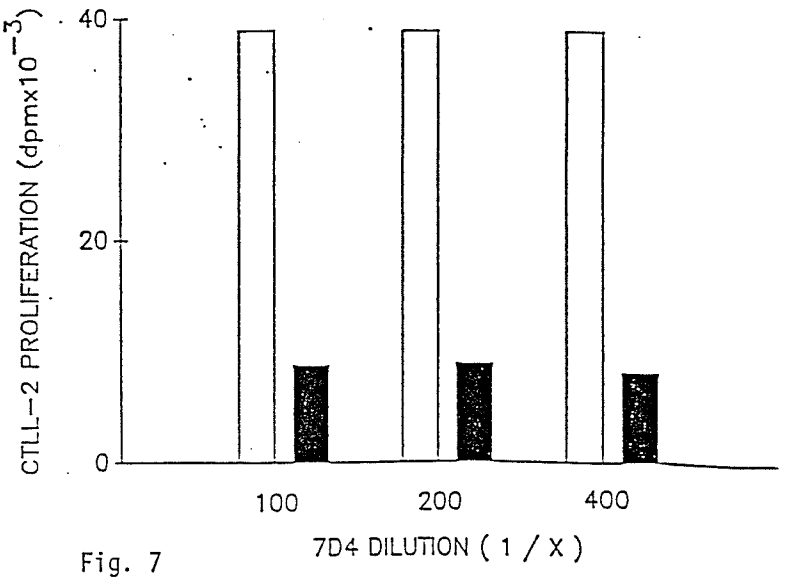

CTLL-2 PROLIFERATION (dpmx10^{-3})
40
20
0
100
200
400
7D4 DILUTION (1 / X)

Fig. 7

Anti-CD3 induced CK gene expression in naive mice

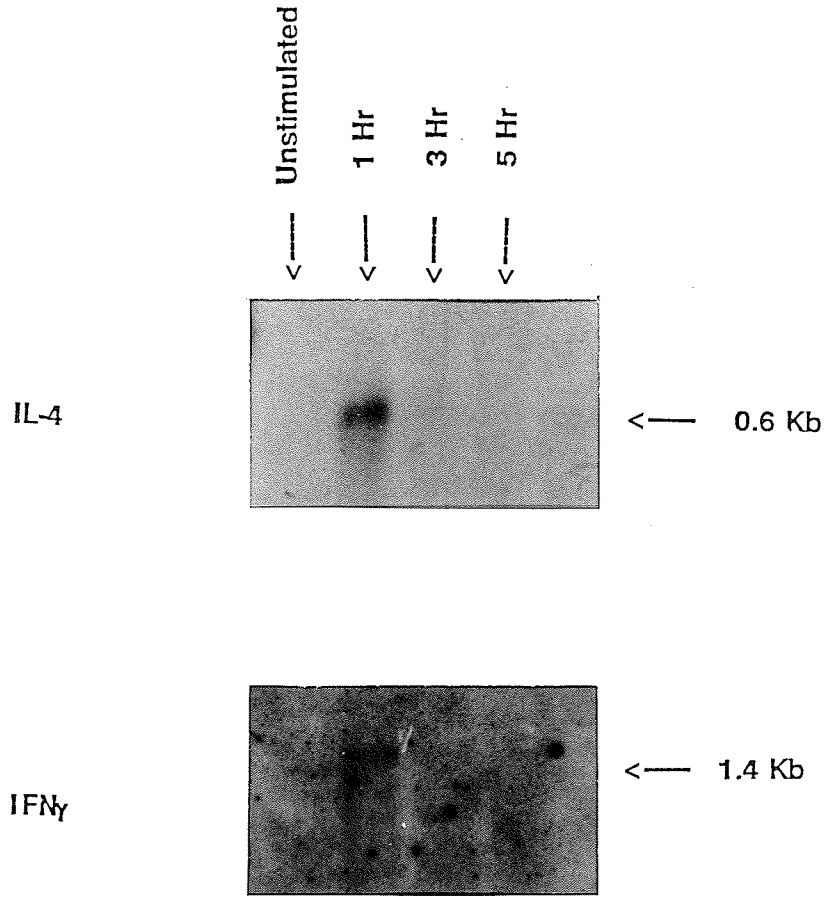
(i) Results following In vivo stimulation

a. mRNA detection

The purpose of our initial experiments was to assess the induction of IL-4, IFNr and IL-2 genes at the mRNA level following polyclonal T cell activation in vivo. It has been reported in the literature that administration of low dose anti-CD3 leads to T cell activation whereas higher doses lead to depletion and dysfunction of T cells (92). In the present study polyclonal T cell activation was accomplished by intravenous administration of low dose (30 ug / mouse) anti-CD3 mAb 145-2C11 to naive C57BL/6 mice (see Materials and Methods). At different time intervals thereafter, mice were sacrificed and total RNA was extracted from their spleen cells. The results obtained by Northern hybridization are shown in Fig. 8 and 9. Equal amount of RNA of different samples was applied in each lane. For IL-4 (Fig.8 top), mRNA signal (~600 bp mRNA species) was detectable within 1 hr of stimulation. By 3 and 5 hr, the signal had decreased to below the limits of detection. No message was detected in unstimulated mice. IFNr mRNA (~1.4 Kb) showed a similar expression pattern, being detectable at 1 hr after stimulation and undetectable thereafter, as shown in Fig. 8 (bottom). In contrast, IL-2

Fig. 8: Northern analysis of IL-4 and IFNr gene expression after stimulation with anti-CD3 *in vivo*

C57BL/6 mice sacrificed 0-5 hours after polyclonal stimulation *in vivo* with 30 ug 145-2C11 mAb i.v.. Total cellular RNA was prepared from stimulated and unstimulated spleen cells by guanidinium-isothiocyanate / CsCl ultracentrifugation. 30 ug RNA was applied in each lane. cDNA inserts of IL-4 and IFNr were used as probes. The IL-4 blot (top) was stripped and reprobed with an IFNr insert (bottom).



In vivo stimulation

Fig. 8

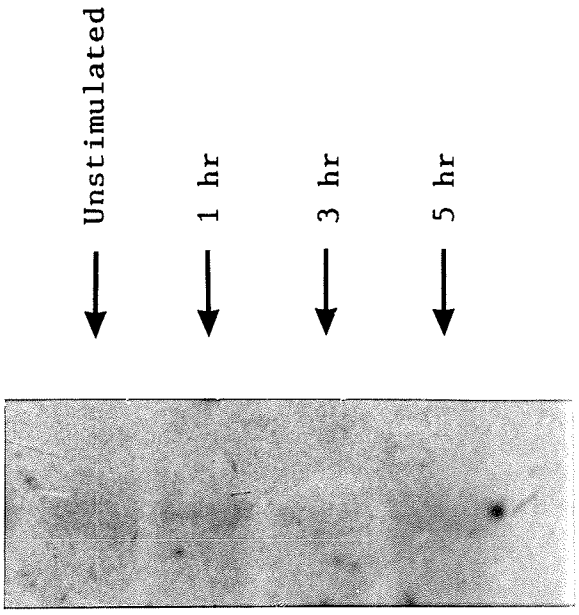
displayed a different expression feature from IL-4 and IFNr, as seen in Fig. 9. The mRNA (900 bp) was detectable in unstimulated and 1, 3 and 5 hr stimulated RNA samples, indicating constitutive IL-2 expression in the population studied, independent of anti-CD3 stimulation. These results suggest to us that, following anti-CD3 stimulation *in vivo*, IL-4 and IFNr genes are induced rapidly in a co-ordinated manner, although IL-2 gene stays unaffected, implying the control mechanisms of transcription of IL-4, IFNr genes and IL-2 gene might differ.

b. Protein production

Reports have been published demonstrating that the expression of a CK mRNA may not necessarily reflect production of a biologically active protein product. For example, Assoian *et al* have found the transforming growth factor beta (TGF-β) mRNA was constitutively expressed in unstimulated and stimulated human peripheral monocytes at a similar level, while TGF-β peptide was only secreted from the activated macrophages (21). Although two unsuccessful attempts were made to identify post-transcriptional regulation of IL-4 genes, Mohler and Butler studying picryl chloride (PCL) induced IL-2 and IL-4 mRNA expression in mice, were unable to detect IL-4 protein production using bioassays with HT-2 (for IL-4 and IL-2) and 11.6 (for IL-4) cells as indicators despite substantial IL-4 mRNA produc-

Fig. 9: Northern analysis of IL-2 gene expression after anti-CD3 stimulation in vivo

RNA was extracted from the same samples by the same method as Fig. 8 and applied 30 ug / lane. The membrane was hybridized to IL-2 cDNA insert probe.



IL-2

Fig. 9

tion (22).

In order to examine whether IL-4, IFNr and IL-2 CK mRNAs induced in vivo were capable of translating into biologically active proteins, it was necessary to carry out specific bioassays of the three CKs. In these experiments, naive C57BL/6 mice were stimulated in the same way with a single dose of 30 ug of 145-2C11 i.v. as before. 30 minutes to 6 hr later, subgroups of mice were sacrificed and their spleen cells were cultured overnight in the absence of in vitro stimulus. The resulting culture supernatants were harvested and evaluated in IL-4, IFNr and IL-2 specific bioassays.

For IL-4 (shown in Fig.10) and IFNr (Table 2), CK protein production was readily detectable in repeated experiments from cells removed between 45 min to 120 min after in vivo stimulation. There was no activity detectable in cells cultured from unstimulated mice. In contrast, IL-2 production was not detectable in any of the samples (data not shown), including unstimulated mice, again showing an independent expression pattern similar to that obtained above. The discrepancy of IL-2 mRNA synthesis and protein production correlates with the literature, which might reflect a high rate of consumption of IL-2 in vivo.

The CK bioassay results indicate, with anti-CD3 in vivo stimulation

Fig. 10: IL-4 production following anti-CD3 stimulation *in vivo*

Naive C57BL/6 mice were injected with anti-CD3 mAb i.v., sacrificed at the times indicated and their spleen cells were cultured for 12-24 hr without additional *in vitro* stimulation. The culture supernatants were assayed for IL-4 activity. mAb 11B11 (anti-IL-4) was used at 0.8 ug / well in the assay as indicated and could block the proliferation to background levels indicating that the CT.4S proliferation was specifically due to IL-4.

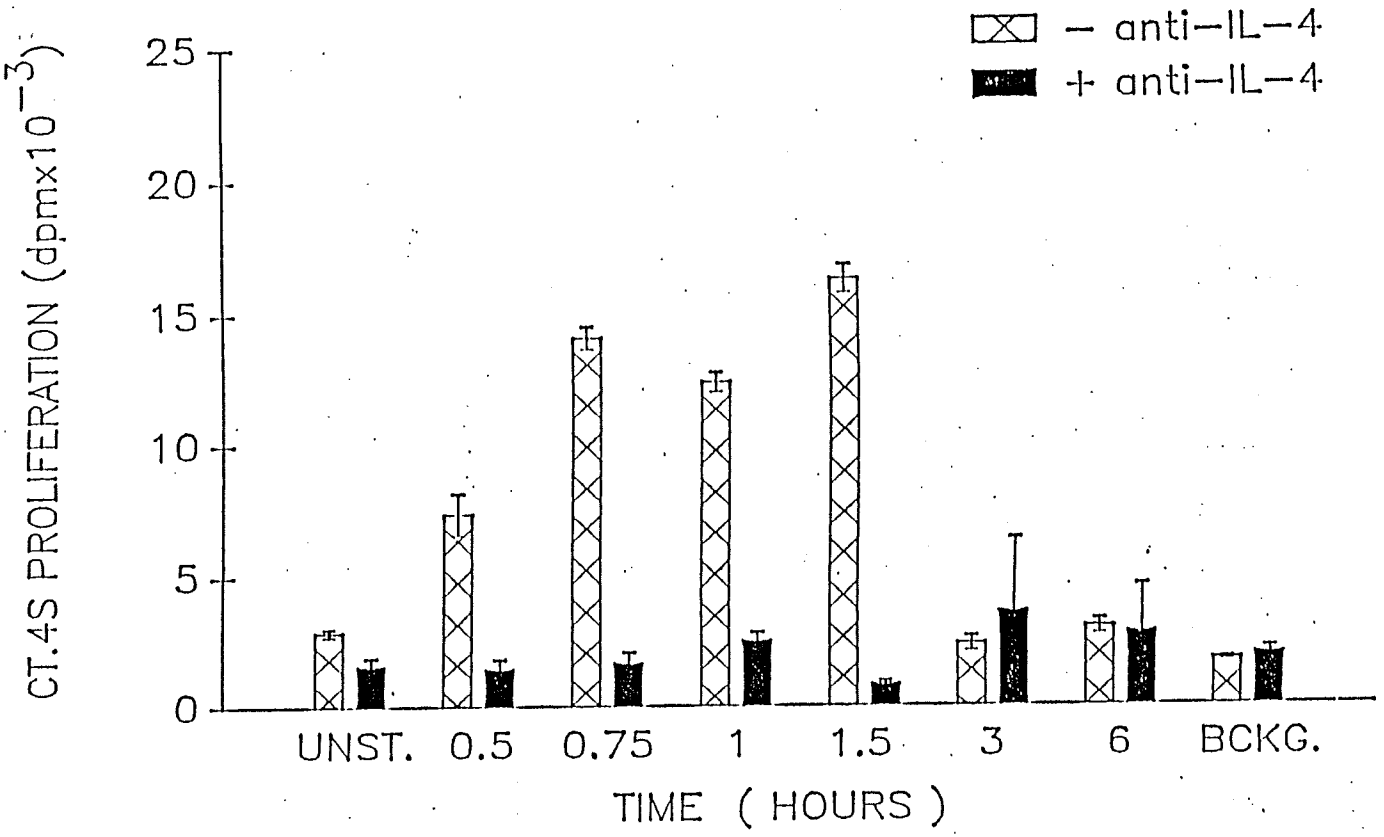


Fig. 10 IN VIVO STIMULATION

Table 2: Demonstration of IFNr protein production following mAb anti-CD3 stimulation *in vivo*

C57BL/6 mice were administered anti-CD3 mAb (i.v.), sacrificed at the times indicated and their spleen cells were cultured for 12 to 24 hr with no additional *in vitro* stimulus. The supernatants were tested for IFNr activity in the bioassay. IFNr was measured as *inhibition* of the proliferation of WEHI 279 indicator cells. Culture supernatants from unstimulated mice were used as negative controls.

Table 2

In vivo stimulus		*In vitro* stimulus	WEHI-279 Proliferation*
Unstimulated	0 hr	None	265,800 ± 25,989
Anti-CD3	0.5 hr	None	202,655 ± 9,972
	0.75 hr	None	12,744 ± 2,510
	1.5 hr	None	9,200 ± 1,545
	3 hr	None	132,300 ± 4,529
	6 hr	None	195,419 ± 9,464

* dpm ± standard deviation.

of naive mice, that functional IL-4 and IFNr protein products coexist with their mRNA expression, whereas this is not the case for IL-2. IL-2 protein may be synthesized, but fast metabolized in vivo so that it was not detectable by in vitro bioassays. Taken together, these results were interpreted as supporting the existence of mature $CD3^+$ T cells in the naive murine T cell repertoire which are capable of IL-4 and IFNr production, without a requirement for extended periods of culture and multiple cycles of stimulation.

(ii) Results following In vitro stimulation

Having obtained results displaying features of IL-4, IFNr and IL-2 gene expression under anti-CD3 stimulation in vivo, the next part of this study was to explore whether the in vivo results also applied to an in vitro stimulation condition with anti-CD3. Specifically, to determine if polyclonal activation of naive spleen cell populations in vitro leads to similarly rapid kinetics in CK gene expression or if, as previously reported, extended periods of culture and stimulation are required .

a. mRNA detection

Naive C57BL/6 mouse spleen cells were stimulated for time periods from 3 to 18 hr with anti-CD3 immobilized on the plastic surface as

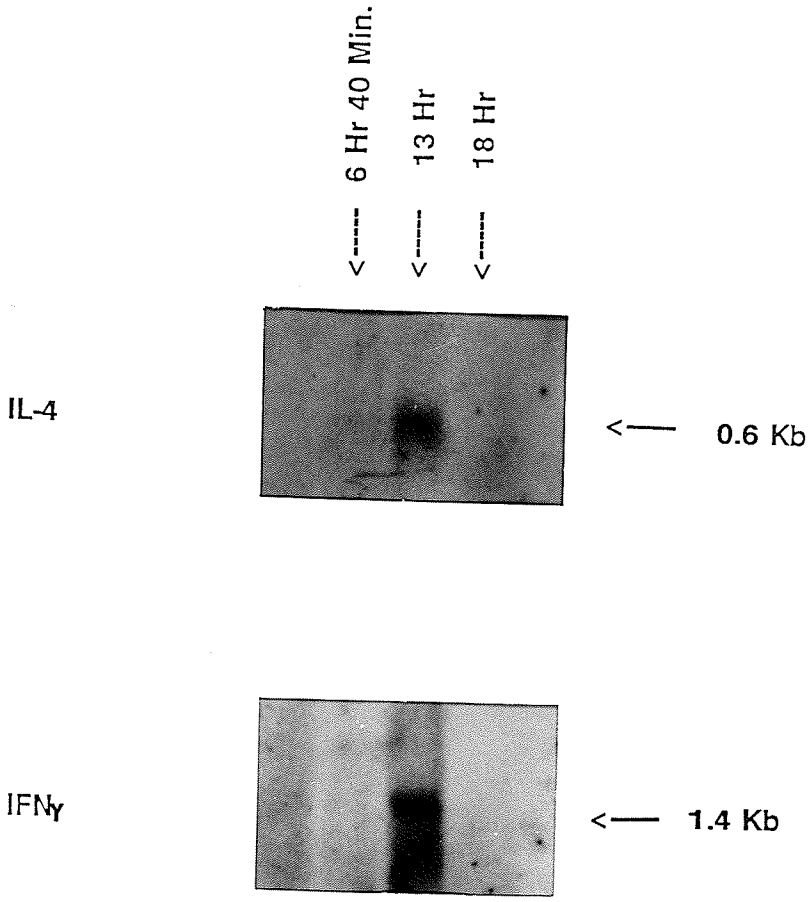
described in Materials and Methods. Northern hybridization was used to assess mRNA synthesis of the three CKs. Total RNA was extracted from the 145-2C11 stimulated cells. The results of Northern hybridization are displayed in Fig 11. As shown, both IL-4 (top) and IFNr (bottom) mRNA signals were detectable in RNA obtained from anti-CD3 stimulated naive spleen cells at 13 hr after stimulation. There was no signal detectable at 6 hr 40 min and 18 hr. Replicate experiments demonstrated there was no mRNA signal detectable at 3, 6 hr and samples from unstimulated cultures for both IL-4 and IFNr gene expression (data not shown). These results indicated that anti-CD3 stimulation of naive C57BL/6 mice in vitro resulted in induction of IL-4 and IFNr mRNA transcription much later than upon in vivo situation (13 hr vs 1 hr). However this expression kept its transient nature and again showed a synchronous manner for both IL-4 and IFNr genes as in vivo. These findings implied that a basic difference might exist in the process leading to T cell activation between anti-CD3 in vivo and in vitro stimulation. But this difference could not change the nature of the IL-4 and IFNr gene expression, i.e. they still showed to be transient and simultaneous.

b. Protein production

The question of whether biologically active IL-4, IFNr and IL-2 were synthesized in parallel to their mRNA transcripts was also addressed

Fig. 11: Northern analysis of IL-4 and IFNr gene expression after stimulation with anti-CD3 *in vitro*.

Naive C57BL/6 mouse spleen cells were cultured at 10 x 10^6 / ml with anti-CD3 mAb precoated onto 24-well plates (10-15 ug / well) for different times indicated. Total RNA was extracted from the cells. 30 ug RNA per lane was applied. cDNA inserts of IL-4 and IFNr were used as probes. The IL-4 blot (top) was stripped and reprobed with an IFNr insert (bottom).



In vitro stimulation

Fig. 11

in in vitro experiments. Here, naive mouse spleen cells were cultured with anti-CD3 immobilized on the surface of 24-well plates (see Materials and Methods) for 3 to 24 hr. The various culture supernatants were collected and assessed for the three CK activity in the CK specific bioassays. The assay results are displayed in Table 3. As shown, biologically active IL-4, IFNr and IL-2 production was remarkably increased after 12 hr in vitro stimulation with the mAb, correlating with the expression of their transcripts. Production remained at maximum within 18 hr after stimulation and slightly decreased by 24 hr, which might represent a biological metabolism of the CKs. This experiment was repeated three times with similar results. A noticable difference for in vitro stimulated samples was that IL-2 was also readily detectable, unlike in vivo samples, representing another difference of in vivo and in vitro situations. Whether this difference reflects a basic biological difference between different modes of T cell activation or differences in assays used remains unclear.

c.Ab blocking assays

The specificities of IL-4 and IFNr assays for in vitro stimulated samples were also tested. As shown in Fig. 12, the anti-IL-4 mAb 11B11 could completely neutralize the IL-4 growth effect on CT.4S indicator cells, indicating that the stimulation reported in Table 3

Table 3: Demonstratration of IL-4, IFNr and IL-2 protein production with anti-CD3 stimulation in vitro.

Naive C57BL/6 mouse spleen cells were cultured at $10x10^6$ /ml with anti-CD3 mAb precoated onto culture plates (24-well) for different hours as indicated. The supernatants were tested in bioassays for IL-4, IFNr and IL-2 activities. IL-4 and IL-2 are measured as the proliferation of CT.4S and HT-2 indicator cells and IFNr as inhibition of the proliferation of WEHI-279 indicator cells. Culture supernatants from in vitro unstimulated mice cultured for the same periods of time were used as negative controls.

Table 3

In vivo stimulus	In vitro stimulus	LK production		
		IL-4	IFNr	IL-2
		(dpm +/- SD)		
None	Unstimulated	5,145 ± 1,021	294,675 ± 17,298	2,731 ± 154
None	Anti-CD3 3 hr	5,715 ± 83	311,763 ± 6,056	4,606 ± 1,148
None	6 hr	8,703 ± 1,441	274,724 ± 7,796	9,077 ± 1,390
None	12 hr	62,196 ± 81	88,000 ± 2,604	181,746 ± 4,524
None	13 hr	60,927 ± 5,770	87,646 ± 1,825	193,802 ± 779
None	18 hr	75,553 ± 1,789	73,918 ± 2,914	247,375 ± 5,484
None	24 hr	47,631 ± 610	63,744 ± 1,577	184,754 ± 4,335

Fig. 12: The specificity of IL-4 bioassay of *in vitro* experiments

mAb 11B11 (anti-IL-4) was used (0.8 ug / well) in anti-CD3 *in vitro* stimulated supernatants in the assay as indicated and could block the CT.4S proliferation to background levels, demonstrating the proliferation was due to IL-4 specifically.

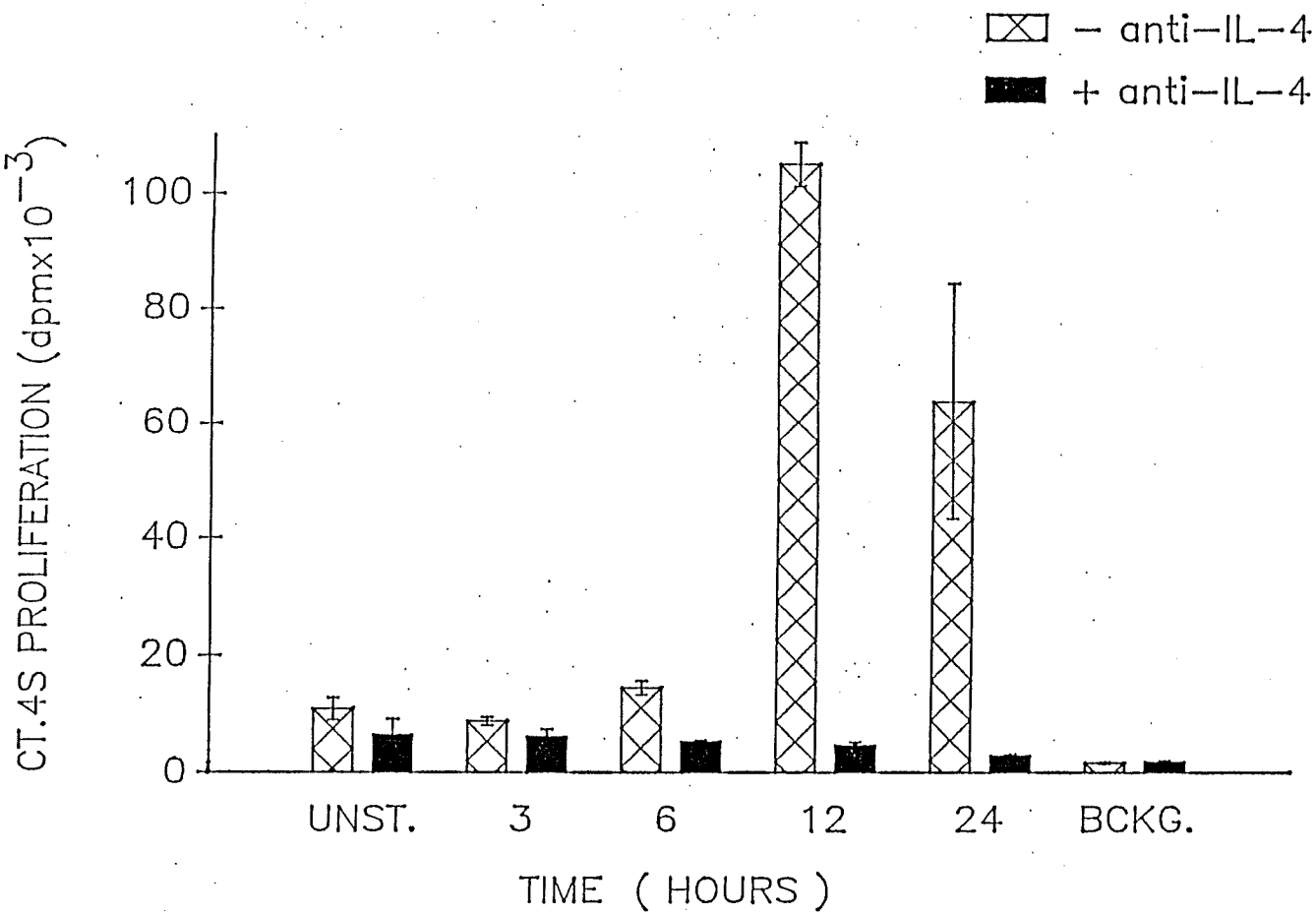

– anti–IL–4
+ anti–IL–4
CT.4S PROLIFERATION (dpmx10^{-3})
100
80
60
40
20
0
UNST.
3
6
12
24
BCKG.
TIME (HOURS)
IN VITRO STIMULATION

Fig. 12

is attributable to IL-4 synthesis after 12 hours of in vitro activation of spleen cells from naive mice with anti-CD3 mAb.

Similarly, the anti-IFNr mAb neutralization results, shown in Fig. 13 indicate the bioassay was IFNr specific and thus that IFNr was also synthesized after 12 hours of in vitro activation of naive spleen cell populations.

Ag (OA, OA-POL) induced CK gene expression

Having established that cells capable of secreting IL-4 are a normal component of the in vivo repertoire, we next examined the production of several cytokines which are of relevance to the development and regulation of allergic responses. Some preliminary results were obtained from studies of allergen stimulated IL-4, IFNr and IL-2 gene expression following in vivo immunization with protein allergen.

(i) OA stimulation

At low concentrations and using appropriate adjuvants, ovalbumin (OA) elicits strong IgE responses in most strains of inbred mice. Here, high responder, naive C57BL/6 mice were injected with 2 ug OA-alum (see Materials and Methods) per mouse, i.p. and, after 4

Fig. 13: The specificity of IFNr assay of *in vitro* experiments

mAb XMG 1.2 (anti-IFNr) was added into anti-CD3 *in vitro* stimulated supernatants in the assay as shown and could block the WEHI proliferation inhibition effect of IFNr in the test supernatants, demonstrating the specificity of IFNr assays.

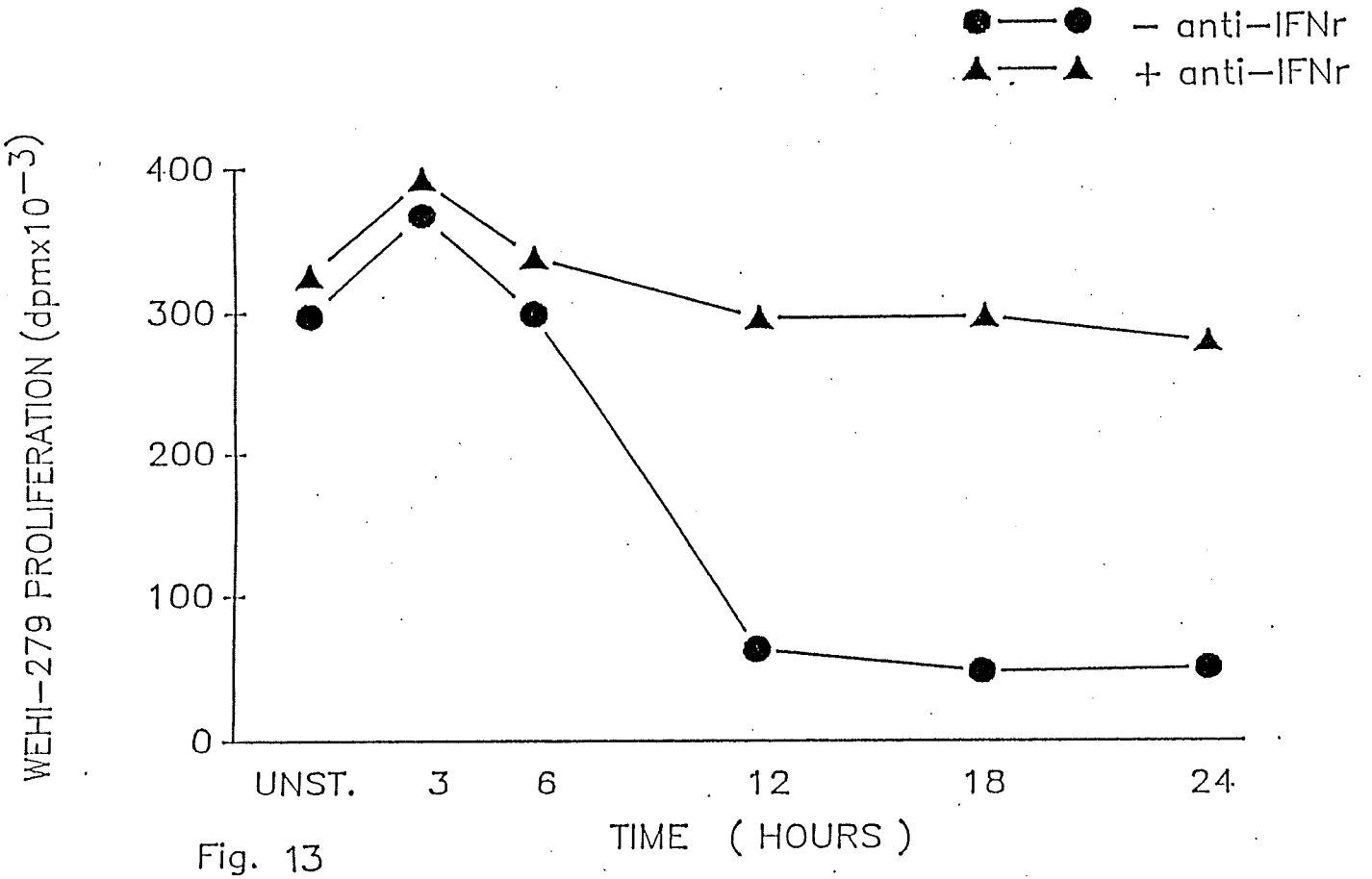

– anti–IFNr
+ anti–IFNr
WEHI–279 PROLIFERATION (dpmx10^{-3})
400
300
200
100
0
UNST.
3
6
12
18
24
TIME (HOURS)

Fig. 13

days, were killed. Spleen cells obtained from these mice were cultured *in vitro* with and without OA as secondary stimulus for 12 to 18 hr. The resulting culture supernatants were assayed for IL-4, IFNr and IL-2 activities. Results are shown in Table 4. IL-4, IFNr and IL-2 production was increased simultaneously with stimulation of OA *in vivo* and *in vitro*. There was no difference in IL-4 and IFNr production between the two different secondary stimulation times. IL-2 production in response to OA was slightly increased in the 18 hr stimulation compared with 12 hr. These results showed that following OA-alum stimulation *in vivo* and restimulation with OA alone *in vitro*, there was an increased production of all three CKs by T cells from immunized mice.

(ii) OA and OA-POL stimulation

Previously published data (31) indicate that administration of a glutaraldehyde polymerized allergen (termed OA-POL) leads to $CD4^+$ T cell-dependent inhibition of naive and ongoing IgE response by at least 90 % with greater than 1,000 fold increases in allergen-specific IgG_{2a} synthesis. Administration of anti-IFNr mAb XMG 1.2 to these mice largely abolished both these effects, suggesting that the dominant T cell CK response to the native Ag *in vivo* was altered as a result of exposure to the chemically modified form. In order to directly examine IL-4 and IFNr production under these

Table 4: OA ($Al(OH)_3$) immunization induced IL-4, IFNr and IL-2 production

OA primed mice were killed on day 4 and spleen cells were cultured with the presence of the secondary OA stimulation (1 mg/ml final) in vitro for 12 to 18 hr. The culture supernatants were assayed for CK production (IL-4, IL-2, proliferation of indicator cells; IFNr, inhibition of proliferation). The supernatant from unstimulated mice was used as the negative control.

Table 4

Secondary OA stimulation (1 mg/ml)	LK production (dpm +/- SD)		
	IL-4	IFNr	IL-2
12 hr	11,808 ± 1,491	92,615 ± 4,807	58,135 ± 11,465
18 hr	12,563 ± 412	89,490 ± 1,279	104,452 ± 8,294
Unstimulated	5,230 ± 1,180	294,239 ± 2,0214	2,754 ± 51

conditions, the CK mRNA expression study was conducted with the sensitive cell blot analysis (see Materials and Methods). C57BL/6 naive mice were separated into four groups with different treatment on days -16, -14 and -12. A: 80 ug OA-POL (in NaCl)/mouse, 3 injections, i.p., as described in Materials and Methods. B: 80 ug unmodified OA (in NaCl, no alum) / mouse, 3 injections, i.p.. C: 2 ug OA-alum/mouse on day 0. D: resting mice as control group, no treatment. On day 0, mice were challenged with 2 ug OA-alum/mouse. Mice from each group were sacrificed on days 3, 5 and 7 and spleen cells were T cell-enriched by panning with sheep-anti-mouse Ig (see Materials and Methods). The enriched T cells were used in cell blots directly and hybridized with IL-4, IL-2 and IFNr cDNA probes.

The IL-4 mRNA expression patterns on day 3, 5 and 7 post-immunization are shown in Fig. 14 and the corresponding densitometry scanning in Fig. 15. The positive signals can be seen between 2×10^5 to 2×10^6 cells / spot. Group A (2×10^6 cells / spot) has markedly less IL-4 mRNA production than groups B or C and in fact is essentially equivalent in terms of IL-4 mRNA synthesis to cultures of fresh, unstimulated mice or to the negative control (HT-2 cells). By day 7, IL-4 mRNA production had dropped to background levels for groups A, B and D. Similarly, IL-2 production in the OA-POL treated, OA (alum) immunized groups (group A) was markedly lower than that in untreated, OA (alum) immunized controls

Fig. 14: IL-4 gene expression patterns in Ag stimulated mice

Cell blot analysis. T-enriched spleen cells from group A, B, C and D mice, on different days of treatment (d 3 top, d 5 middle, d 7 bottom) were loaded on each spot with 2 x 10^3 to 2 x 10^6 cells. Anti-CD3 stimulated (same as before) cells were positive controls (p α-CD3: panned cells; up α-CD3: unpanned cells, as a control for the panning procedure itself). HT-2 cells were negative controls. The treated membranes were hybridized to IL-4 insert.

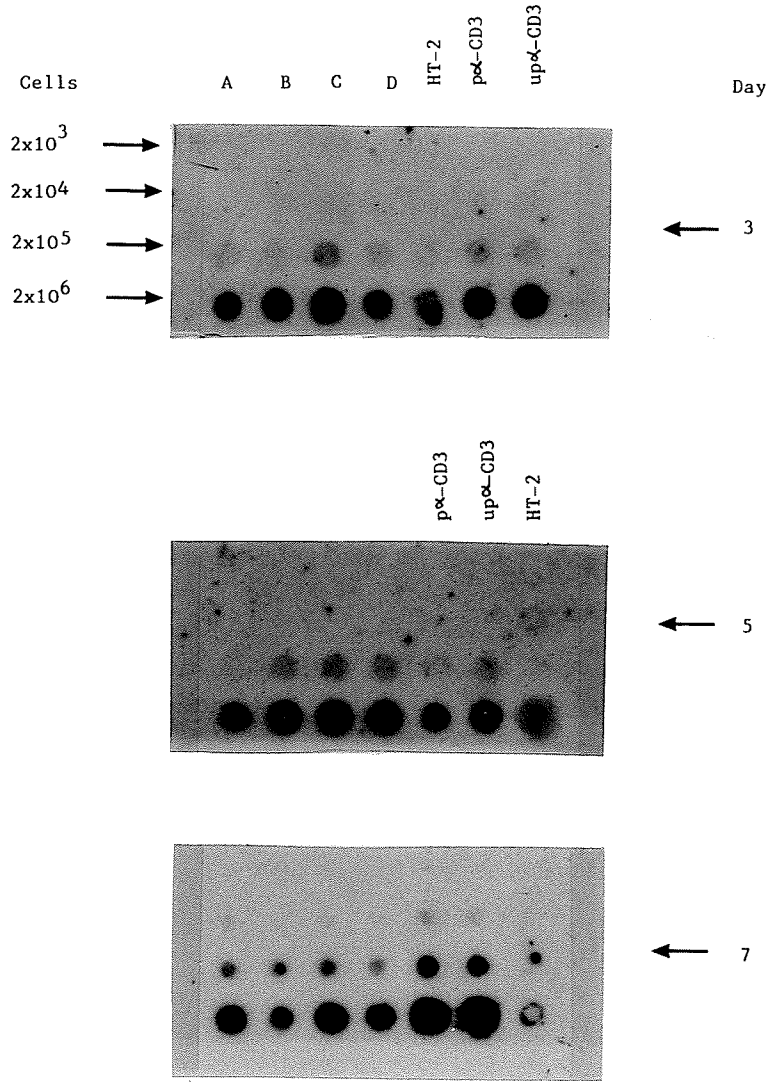

Cells
A
B
C
D
HT-2
pα-CD3
upα-CD3
Day
2x10^3
2x10^4
2x10^5
2x10^6
3
pα-CD3
upα-CD3
HT-2
5
7
IL-4

Fig. 14

Fig. 15: Densitometry scanning (relative, for slots) of Fig. 14 (row 2×10^5 and 2×10^6)

2×10^5 2×10^6

Day 3

Day 5

IL-4

Day 7

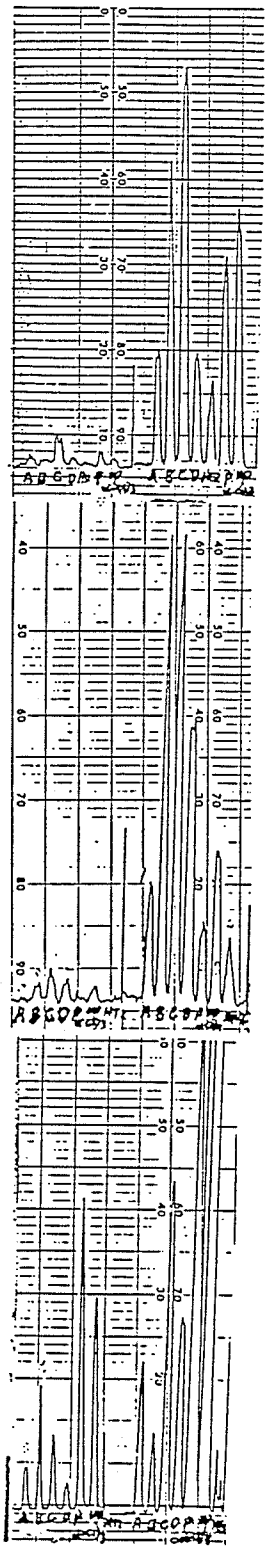


Fig. 15

(group C) at 3 and 5 days post-immunization (Fig. 18 and 19).

In contrast to the above, examination of IFNr gene expression late in the response (d 7, Fig. 16 and 17) is suggestive of markedly higher IFNr production in OA-POL treated groups. IFNr mRNA production in unstimulated (group D), OA treated, OA (alum) immunized groups (group C) is 25-75 % lower than that of OA-POL pretreated OA (alum) immunized groups.

Fig. 16: IFNr gene expression patterns in Ag stimulated mice

Cell blot analysis. Same experimental design as Fig. 14. The cDNA probe was IFNr insert.

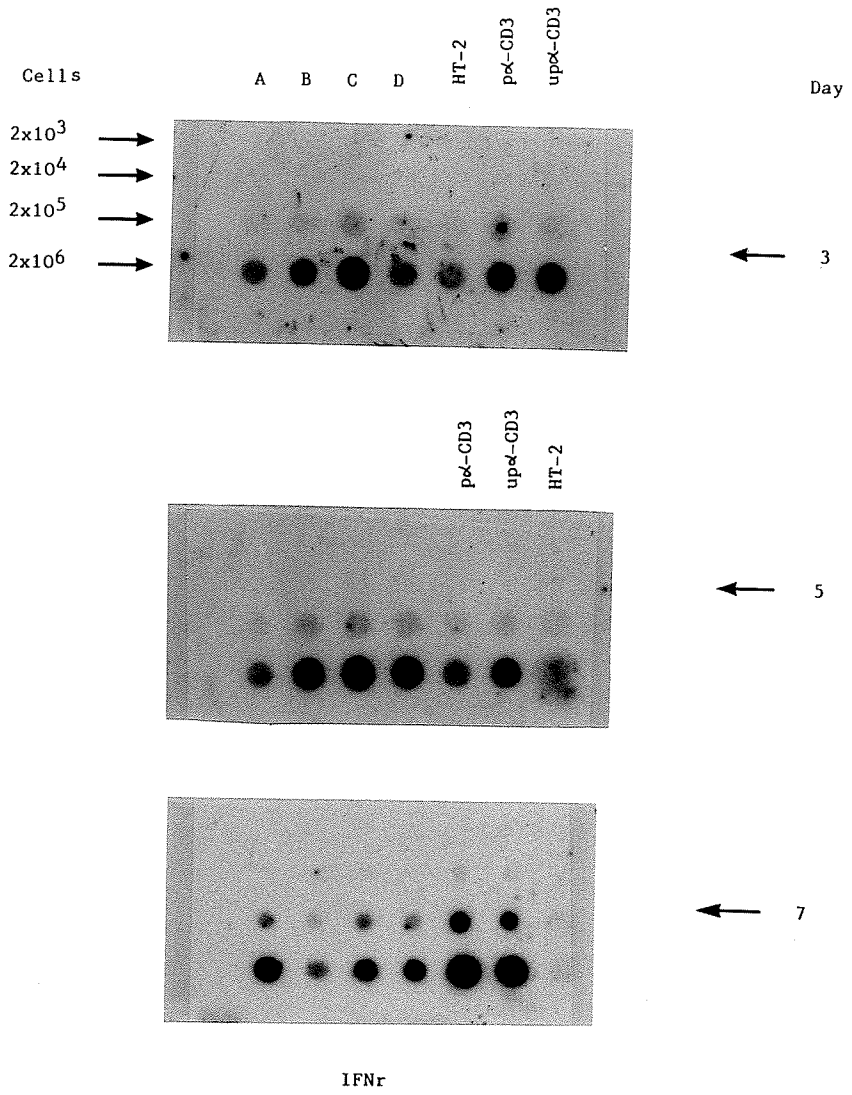

Cells
A
B
C
D
HT-2
pα-CD3
upα-CD3
Day
2x10^3
2x10^4
2x10^5
2x10^6
3
pα-CD3
upα-CD3
HT-2
5
7
IFNr

Fig. 16

Fig. 17: Densitometry scanning of Fig. 16.

2×10^5 2×10^6

Day 3

Day 5

IFNr

Day 7

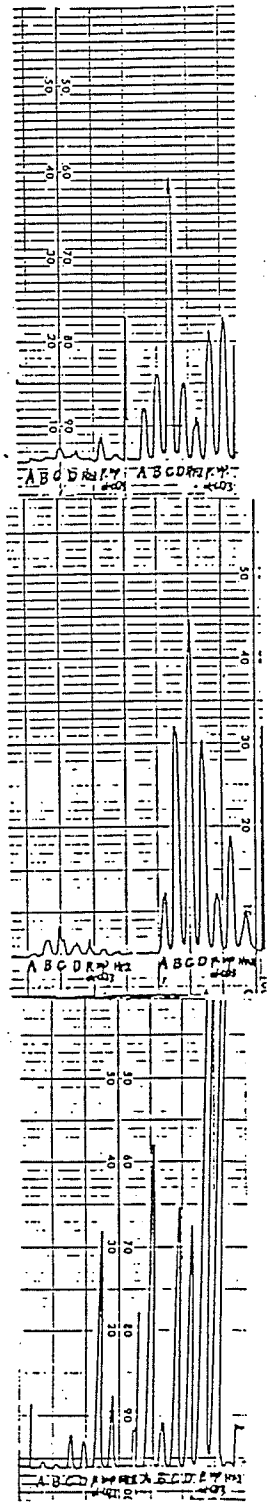


Fig. 17

Fig. 18: The IL-2 gene expression patterns in Ag stimulated mice

Cell blot analysis. Experimental design as in Fig. 14. IL-2 insert was the hybridization probe.

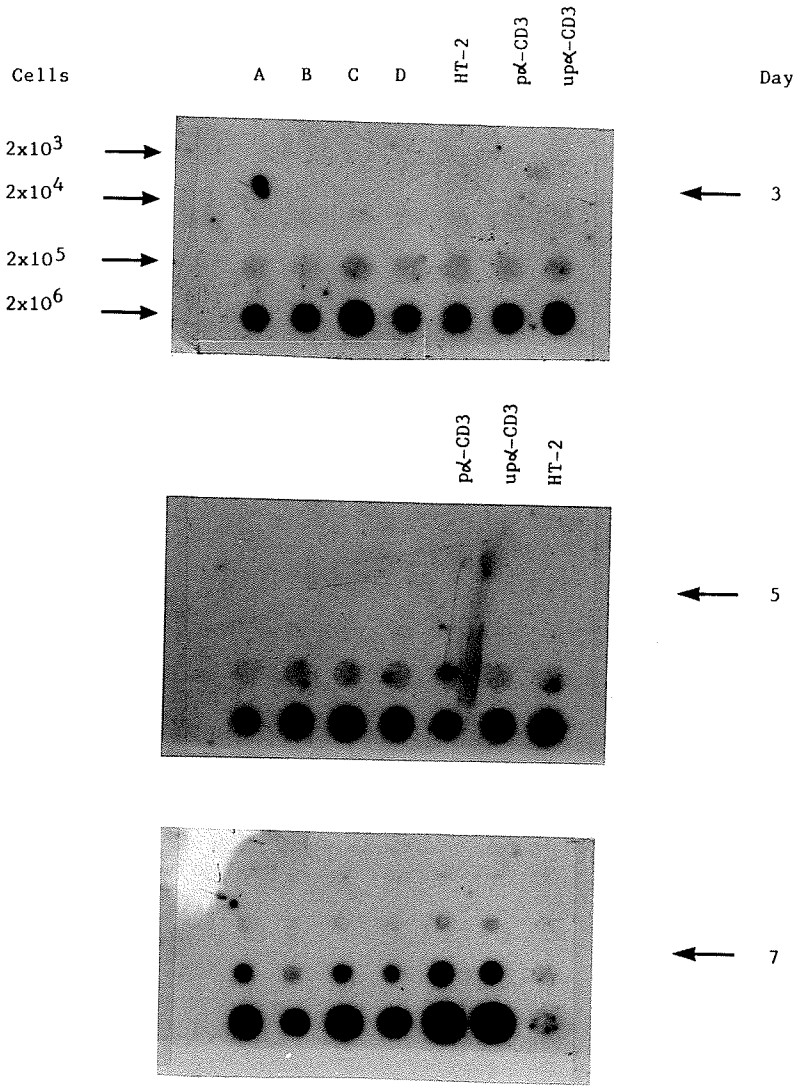

Cells
A
B
C
D
HT-2
pα-CD3
upα-CD3
Day
2x10^3
2x10^4
2x10^5
2x10^6
3
pα-CD3
upα-CD3
HT-2
5
7
IL-2

Fig. 18

Fig. 19: Densitometry scanning of Fig. 18

2×10^5 2×10^6

Day 3

Day 5

IL-2

Day 7

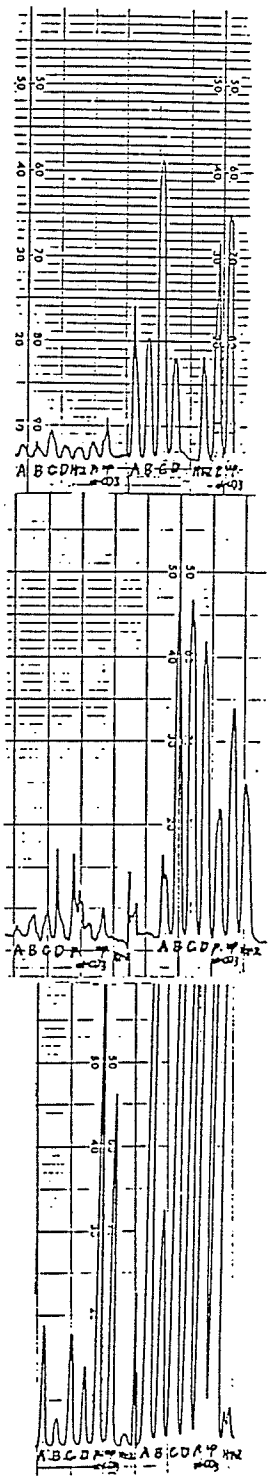


Fig. 19

DISCUSSION

In order to study the expression of IL-4, IFNr and IL-2 genes in the naive and Ag primed T cell compartment, various methods of *in vivo* and *in vitro* stimulation were employed in this study. CK gene expression was assessed at both mRNA level and protein level by several different detection methods.

There are a variety of detection methods currently being used in CK gene studies. However, with various technical limitations, each method usually possesses specific advantages and disadvantages for a particular study as described in the Introduction (4, 44). Therefore, to accomplish the research objectives in a study successfully, one should consider a combined utilization of different detection methods for gene products (mRNA and protein), based on the type of tissue to be used and the level of the CK gene expression (4).

The detection methods chosen for this study include Northern blotting, RNA dot blotting, cell blotting and CK specific bioassays. The selection of assay must take into account the fact that most CK genes are expressed non-constitutively and at low abundance. Due to the imperfection of any one assay, these approaches are mutually dependant in that Northern, dot and cell blots are highly specific when used with appropriate CK specific cDNA probes, but are limited

by the transient nature of CK gene expression. This problem can only be circumvented by frequent sampling of the cell population of interest. Bioassays are highly sensitive when CK specific conditions are established by using sensitive indicator cell lines in combination with appropriate CK specific Abs. Interpretation of bioassays is limited by the fact that one measures the net CK production i.e. the amount produced minus the amount consumed in culture prior to bioassay. Therefore the combined application of the above approaches is necessary to obtain reliable results of IL-4, IFNr and IL-2 gene expression study at both mRNA and protein levels.

In mice stimulated with low dose anti-CD3 in vivo , IL-4 and IFNr mRNAs were rapidly and transiently induced in a co-ordinate way, being detectable on Northern blot within 1 hr of stimulation in vivo (Fig. 8). As such, these results imply there is a rapid upregulation effect of anti-CD3 mAb stimulation on mRNA transcription of IL-4 and IFNr genes without a requirment for protracted in vitro culture or multiple cycles of antigenic or polyclonal stimulation. This observation stands in marked contrast to the widely held view (23, 113,114) that T cells " with the potential to become IL-4 secreting cells exist in vivo in the form of precursors requiring stimulation and several days of culture as well as restimulation with mitogen or antigen before they become detectable

as lymphokine secreting cells." (23). Reports similar to our own, demonstrating transient expression of IL-4 and IFNr mRNA following polyclonal T cell activation, have recently appeared in the literature (92,93). Although no differences could be detected in IL-4 mRNA expression in normal naive mice, specific pathogen free mice or germ-free mice (92), the potential for previous T cell priming (by environmental Ags) remains. Our observations that unstimulated spleen cell populations do not demonstrate detectable IL-4 mRNA is the evidence only that active infections are not underway. Priming of T cell populations by previous infections would not be evident in unstimulated groups as constitutive IL-4 or IFNr expression has not been reported to date. An improved approach for future studies wold involve Ag specific T cell activation (using an allergen for which the mice are demonstrably naive) in preference to polyclonal activation which activates naive and memory T cells both.

There were no differential regulatory effects detected on IL-4 and IFNr gene transcription with anti-CD3 stimulation in this study. The two were expressed at the same time and for similar duration. The IL-2 gene expression showed a different characteristic (Fig. 9). It was expressed in both stimulated and unstimulated mice and was expressed at the same level in these mice without being apparently influenced by the anti-CD3 stimulation. This suggests that IL-2

gene transcription might be controlled and regulated by different mechanisms from IL-4 and IFNr genes and that IL-2 might be a basic requirement for the maturation of Th 2 cells to secrete IL-4 (24) and Th 1 cells to secrete IFNr. Similar results of IL-2 gene being regulated differentially from IFNr gene could also be found in the published literature (1) but more extensive work would be required to confirm this point.

A second important finding concerns the relationship between CK mRNA and protein production following polyclonal and antigen specific T cell activation. As stated above, evidence has been found (21,22) that mRNA expression of certain cytokines (e.g. TGF-β) may not result in their functional protein products due to a variety of reasons: the mRNA being untranslated, biologically inactive or rapidly metabolized. Recent studies indicating IL-4 and IFNr mRNA production by naive mice following Ag specific or polyclonal activation either did not examine or were unable to demonstrate IL-4 <u>protein</u> production, raising the possibility that the IL-4 mRNA observed in these recent studies was not capable of being translated (21, 22). However this study, looking at IL-4 and IFNr synthesis obtained following anti-CD3 stimulation of naive mice, has demonstrated functional protein activities of IL-4 and IFNr (Fig.10, Table 2) in correspondence to their mRNA expression, indicating that the IL-4 and IFNr mRNAs were translated into

biologically active IL-4 and IFNr proteins which are readily detectable in the CK bioassay systems used here. The reason for our ability to demonstrate IL-4 under conditions used unsuccessfully by others is probably due to the use of the highly IL-4 sensitive CT.4S bioassay instead of markedly less sensitive assays for IL-4 used by previous investigators such as HT-2 proliferation or MHC Class II expression by BCL1 cells (91).

Interestingly, there was no significant detectable IL-2 functional protein detectable from 145-2C11 in vivo stimulated mice regardless of the observation of constant mRNA expression of IL-2 observed in all mice (stimulated and unstimulated). This result may indicate that a different post translational fate of IL-2 molecules from IL-4 and IFNr exists. However, it is equally possible that IL-2 was consumed at a high rate in vivo by other activated T cells so that it was not detectable at the protein bioassay level. Further work is required to determine this.

It was a consistant finding that with anti-CD3 stimulation in vitro, the IL-4 and IFNr gene expression was detected only after 13 hr stimulation (Fig, 11), which was much later than the expression induced by in vivo stimulation. This result reveals an essential difference which existed in the two different modes of stimulation: in vivo and in vitro, leading to T cell activation. Our hypothesis

to explain this difference is that the upregulation signal from *in vitro* stimulation was transduced to the IL-4 and IFNr genes much slower than *in vivo* stimulation. What may cause the slow signal transduction under *in vitro* conditions? This phenomenon might be due to the lack of biological environment in *in vitro* situations as well as numerical and topographical changes in TCRs, which could contribute to the slow signal transduction. However an important point is that the nature of the expression of IL-4 and IFNr genes was not changed by the slower T cell activation signal. Both were still turned on rapidly, expressed very briefly and shut off rapidly. The two genes were still regulated in a synchronous and co-ordinate way as observed following *in vivo* stimulation. Alternatively, this may be due to the stress period of the *in vitro* handling to which cells are subjected. Only freshly divided cells generated during 1-6 hr after culture can be activated by anti-CD3.

When using CK specific bioassays to detect functional IL-4, IFNr and IL-2 molecules following *in vitro* stimulation, not only was a large amount of IL-4 and IFNr production detected between 12 to 24 hr after stimulation, but also a tremendous increase of IL-2 production was detected within the same time period, as shown in Table 3. We interpret this result as supporting evidence for the hypothesis of high rate of consumption of IL-2 *in vivo* by showing a relatively longer half-life of IL-2 *in vitro* and being easily detectable in

bioassays, which suggests the turn-over rate of IL-2 is much slower under in vitro conditions than in vivo.

Our results show that a single in vivo or in vitro anti-CD3 stimulus elicits rapid upregulation of IL-4 expression detectable at both the mRNA and the protein level (Fig. 8, 10, 11, Table 3). This is strong evidence that IL-4 secreting T cells in vivo exist not only as precursors but also as mature committed T cells. This conclusion is based on the observation (23) that precursor cells require primary stimulation in vivo (antigenically or polyclonally) as well as restimulation cycles in vitro over many days in order to secrete IL-4, whereas mature T cells do not require restimulation cycles to secrete IL-4 (91). This is important and may be critical in allergic immune responses (Type I Hypersensitivity), since IgE production has been well established to require the presence of IL-4 (53). In addition, IL-4 is also required for B cell growth. Therefore, antigen-specific IL-4 secreting T cells should exist as mature peripheral T cells ready to produce IL-4 on contact with Ag rather than only as precursors which require repeated stimulation to secrete IL-4.

There have been a few papers on anti-CD3 research published in last year (92-94). Although anti-CD3 mAb was also used as stimulator to induce CK expression in these papers, each paper has its own

research focus and different read out systems. None of them did the same systemic study with anti-CD3 administered both *in vivo* and *in vitro* for a series of time periods and studying CK gene expression at both mRNA and protein levels as the present study.

As to Ags OA (allergen) and OA-POL stimulation, the CK gene expression picture was more complex probably involving a higher level of interaction between accessory cells (e.g. APC and B cells) and T cells and other molecules (e.g. adhesion molecules) and factors (e.g. various CKs). No clear cut characteristic features of any CK expression could be concluded at this stage and further study will be required. The discrepancy in the pattern of CK gene expression seen between the polyclonal (anti-CD3) and antigenic stimulation may be due to the difference in number and type of cells they stimulate. The antgenic stimulation may involve mature T cells which will be fewer in number than that of the polyclonally activated T cell populations. However, one phenomenon appeared rather obviously in all of the Ag stimulated results, i.e. if one compares the cell blots of three CKs expressed on the same day, it appeares that IL-4, IFNr and IL-2 CK genes were upregulated and downregulated synchronously in both OA stimulated and OA and OA-POL stimulted experiments at the mRNA level (Fig. 14, 16, 18) as well as the protein level (Table 4). Gauchat *et al* studied CK IL-4, IL-2 and IFNr mRNA expression from human peripheral blood mononuclear cells (PBMNC) from nor-

mal donors and allergic donors induced by different Ags and allergens and also was unable to detect differences in the three CK mRNA levels from normal and allergic donors. IL-4 mRNA expression was not increased in allergic donors (44). Altogether, these data suggest that at the transcriptional level, there may not be significant differences in regulation of various CK genes at least as detectable by the techniques used to date. On the other hand, each CK does have its own biological effects, e.g. high IgE production requires a higher IL-4 level and high IFNr production can inhibit this process. This would not be possible if every CK is only expressed at a constant level relative to one another under any circumstances. Therefore some other post transcriptional (e.g. control of different mRNA half-lives) and post translational factors (e.g. different CK activity controls, directional release of cytokines between interacting cells) may be playing a role in regulating the different overall effects of each CK <u>in vivo</u>.

CONCLUSIONS

The molecular and bioassay methods employed in this study have been demonstrated to be CK specific and highly sensitive.

Anti-CD3 mAb stimulation of naive mice *in vivo* induces rapid and transient IL-4 and IFNr gene expression. This expression peaks one hr after the stimulus.

The functional proteins of IL-4 and IFNr coexist with their mRNA transcripts expressed between 45 min to 3 hr after this stimulus, indicating that IL-4 and IFNr production does indeed occur very rapidly following polyclonal activation *in vivo*.

The IL-4 and IFNr gene expression appears to be regulated synchronously following anti-CD3 stimulation *in vivo*. In contrast, IL-2 gene expression appears constant in all mice, not being upregulated by anti-CD3 mAb stimulation *in vivo*. The protein activity of IL-2 is not detectable under the conditions used regardless of its mRNA expression. Thus, differential regulation of IL-2 gene vs. IL-4 and IFNr genes can be observed with anti-CD3 stimulation *in vivo*.

In vitro stimulation of naive mouse spleen cells with anti-CD3 confirms the rapid induction of IL-4 and IFNr gene expression. This

expression is detectable approximately 13 hr after the stimulus. The functional IL-4 and IFNr protein can also be detected at approximately the same time after stimulation.

The IL-4 and IFNr genes are synchronously regulated by anti-CD3 stimulation *in vitro* but unlike *in vivo* stimulation with 145-2C11, IL-2 protein is detectable after 12 hr stimulation with anti-CD3 *in vitro*.

Although not conclusive, the studies involving Ag specific stimulation *in vivo* and *in vitro* indicates no significant differences in the IL-4, IFNr and IL-2 expression regulation. The pattern of up and down regulation of CK gene expression may differ from that of the anti-CD3 stimulation.

Our study has provided direct evidence for the existence of mature IL-4 secreting T cells *in vivo* in the unstimulated lymphocyte pool.

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