IMMUNOLOGY OF TUBERCULOSIS: NEW DIRECTIONS IN RESEARCH•

GIOVANNA LOMBARDI *, FLAVIA DEL GALLO *, DANIELA VISMARA *, ENZA PICCOLELLA *, VITTORIO COLIZZI **

*Dipartimento di Biologia Cellulare e dello Sviluppo, Sezione di Patologia Sperimentale *, Università 'La Sapienza', Roma*

*Istituto di Microbiologia **, Università degli Studi di Pisa*

More than 100 years ago Robert Koch presented his exciting description of *Mycobacterium tuberculosis*, the parasite responsible for tuberculosis. In this last century, the preparation of the bacille Calmette-Guérin (BCG) vaccine, the development of effective radiodiagnostic procedures and chemotherapeutic agents and, more important in the control of the infection, the improvement of socioeconomic standards, have caused a sharp decline of tuberculosis incidence in the developed countries. However, tuberculosis remains a major uncontrolled health problem in almost all over the world. Recent WHO (World Health Organization) data[4] on the world population lead to the projection of two million deaths from tuberculosis in the year 2000. Furthermore, epidemiological studies carried out in Italy by the *Ministero della Sanità*[35] have pointed out an incidence of 29-145/100,000 cases of tuberculosis in 1982. Considering these epidemiological data and projections, the WHO has passed a plan of research on the immunology of tuberculosis with the aim of successfully applying immunological strategies to the control of this disease.

Experimental studies have only partially made clear the fundamental mechanisms of the immune response to *M. tuberculosis*. The first rational approach was attempted by Koch himself observing that guinea pigs previously infected with *M. tuberculosis* developed an inflammatory skin reaction to a challenge

---

Key-words: Antigen-presenting cells; DNA recombinant vaccine; T cell clones; T suppressor cell soluble products; Tuberculosis.

• These studies were supported by 'WHO Vaccine Development Programme', *Consiglio Nazionale delle Ricerche (CNR), Roma*, Italy (grant n° 85.00875.52) and *Associazione Italiana per la Ricerca sul Cancro (AIRC)* (fellowship to G. L.).

Accepted for publication on November 4, 1986.

La Ricerca Clin. Lab. *17*, 1, 1987.

injection with tuberculin. The lesion did not develop in uninfected animals indicating its specific nature. This first description of delayed-type hypersensitivity (DTH) to mycobacterial antigens has raised several questions concerning the basic mechanisms of the host's response to *Mycobacteria* and other intracellular parasites, i.e. questions regarding also the causative relationship between DTH and acquired antibacterial resistance.

It is now widely accepted that protection against facultative intracellular bacteria as well as DTH are mediated by antigen-specific T lymphocytes[18]. In fact, T cells from *Mycobacteria*-infected donors produce, after *in vitro* antigenic stimulation, multiple lymphokines such as interleukin-2 (IL-2), migration-inhibiting factor (MIF), macrophage-activating factor (MAF) and γ-interferon (IFN). The result of the lymphokine production is the hyperactivation of macrophages which differ from normal macrophages as far as cell size, surface molecules expression[1], lysosomal acid hydrolases, respiratory enzymes and microbicidal activity[39] are concerned. Lymphokine-activated macrophages are therefore the final products responsible for protection, inflammatory reaction and tissue damage.

During the last 10 years, basic immunology has developed new technologies and provided new findings which allow a more detailed analysis of the mechanisms that are the basis of protective immunity and DTH. The development of monoclonal antibodies to T cell differentiation markers and clonotypes[37] and the T cell cloning technology[36] have pointed out that the T cell system consists of different, functionally distinct cellular subpopulations recognizing nominal antigen in the context of class I or class II molecules of the major histocompatibility complex (MHC)[48]. The different T cell subsets and their role in the antibacterial immunity are now key points under investigation in the model of *Listeria monocytogenes*[24] and *M. tuberculosis*[40] infections.

The aim of this review is to give a brief description of the immunology of tuberculosis by providing recent data obtained in our laboratories on the cellular interactions involved in the immune response to *M. tuberculosis.* In particular, the following aspects will be discussed: *i.* the role of B cells in processing and presenting *M. tuberculosis* antigens to T cells; *ii.* the activation and characterization of mycobacterial-specific T lymphocytes; *iii.* the cellular and molecular mechanisms regulating the immune response to *M. tuberculosis.* Finally, a genetic engineering approach to the development of a second generation vaccine against *M. tuberculosis* will be briefly presented.

1. *Role of B lymphocytes in phagocytizing and processing M. tuberculosis*

The ability of macrophages to phagocytize *M. tuberculosis* and then function as antigen-presenting cells (APC) in the induction of mycobacterial-specific T cell responses has been extensively reported both in human and in experimental animals. However, the attention has been recently focused on the ability of some 'classically' defined nonphagocytic cells, such as the lymphoid dendritic cells[20,25], and B cells[6,7,26] to induce many T cell responses *in vitro.* In most of these studies, soluble forms of antigens have been used and the biological role of such cells in the induction of the immune response to intracellular pathogens is unknown. To extend our knowledge of the functional activity of B lymphocytes as APC and to investigate such role in the antitubercular immunity, we examined their ability to phagocytize and process *M. tuberculosis* cells[27].

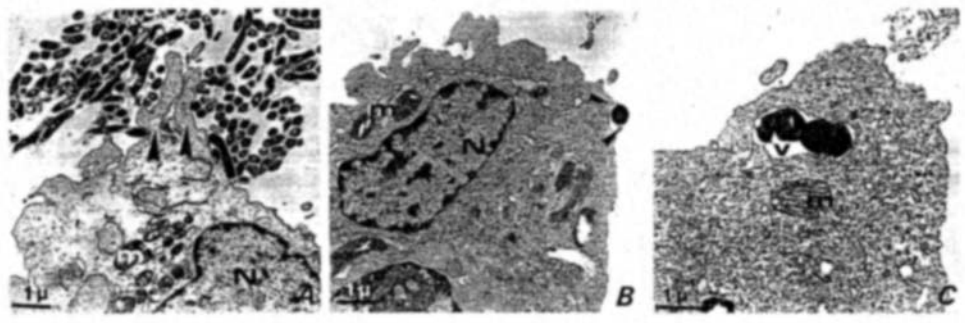

Fig. 1 - Electron micrograph of EBV-B cells at different times of whole-H37Rv incubation. *A*: several mycobacterial cells can be seen outside the cells which often show pseudopodia (arrows) after 2h of incubation; *B*: the arrows indicate an initial invagination of plasma cell membrane partially surrounding a mycobacterial cell; *C*: several mycobacterial cells are visible inside the membrane-bound intracytoplasmic vacuole (v) after 18h of incubation. N = nucleus; m = mitochondria.

For this purpose, human B cells were transformed *in vitro* with the Epstein-Barr virus (EBV) and then used to investigate the following steps of the antigen presentation: *i.* the phagocytosis of the whole *M. tuberculosis* cells; *ii.* the processing of *M. tuberculosis*; *iii.* the presentation of mycobacterial epitopes to T cells.

The capacity of EBV-B cells to phagocytize the whole *M. tuberculosis* cells (H37Rv strain) has been analyzed by electron microscopy at different times of the antigen pulsing. After 2h of incubation, several EBV-B cells showed cytoplasmic elongated projections within clusters of mycobacterial cells. Some mycobacterial cells appeared very close to the plasma membrane which often began to undergo invagination around the bacterium (fig. 1, *A* and *B*). After 18h of incubation, numerous mycobacterial cells are engulfed in enlarged phagosome-like organelles (fig. 1*C*). The presence of lysosomal vacuoles inside the cytoplasm of EBV-B cells has been recently reported by CHU et al.[7] using electron microscopy and by staining the cells with acid phosphatase. Lysosome-like and acid phosphatase-containing vacuoles similar to those seen in monocytes were found in tumor B cells but were absent in resting B cells. The possibility that *in vivo*-activated B cells behave similarly to tumor cells in their antigen presentation has been recently reported[6]. Work is in progress to observe the presence of lysosomes on mycobacterial-activated normal B cells.

The electron microscopy observations have raised the question about the capacity of EBV-B cells to process and present mycobacterial antigen preparations to specific T cells. This point has been investigated by evaluating the ability of both the particulate and soluble forms of antigen to induce the proliferation of mycobacterial-specific T cell clones (TLC). The particulate forms of antigen consisted of the whole *M. tuberculosis* (whole-H37Rv) and of the *M. bovis* strain BCG (whole-BCG) cells, whereas soluble antigens consisted of the purified protein derivative (PPD) and of the soluble extract from *M. tuberculosis* (soluble-H37Rv). Table 1 shows that EBV-B cells present not only the soluble but also the particulate forms of mycobacterial antigen when added to T cell cultures, although less efficiently than peripheral blood mononuclear cells (PBMC).

T cells	PPD	soluble-H37Rv	whole-H37Rv	whole-BCG
		autologous PBMC		
Ew4/7	16,107	57,024	35,656	14,522
Ew4/10	5,647	4,373	4,769	1,264
LL1	16,212	nd	12,892	nd
		autologous EBV-B cells		
DGA1	12,541	10,944	1,475	nd
LL1	6,382	nd	12,648	nd
LB1	8,399	nd	5,077	3,497

PPD-specific T cell lines and clones (10^4 cells/well) were cultured for 4 days with either irradiated (3,000 rads) autologous PBMC (10^5 cells/well) or EBV-B cells (10^4 cells/well). The antigen preparations were added at the beginning of the cultures, while radioactive thymidine (0.5 μCi/well) was added 12h before the end of the cultures. The results were expressed as cpm and the standard deviation was always less than 10%. Background proliferation (no antigen) was subtracted; nd = not determined.

Tab. 1 - Presentation of soluble and particulate mycobacterial antigens by autologous PBMC and EBV-B cells.

The requirement of the processing step for antigen presentation of the whole-H37Rv cells has been analyzed by using chloroquine, an agent which interferes with normal lysosome functions both at the level of macrophages[54] and B tumor cells[17]. For this purpose, EBV-B cells were incubated for 2h with chloroquine and antigen preparations, then washed and cultured for 3 days with TLC. Table 2 shows that the treatment of EBV-B cells with chloroquine inhibits T cell activation when the whole-H37Rv is used, whereas the same treatment only marginally interferes with PPD presentation. Increasing the concentration of chloroquine, also the presentation of PPD is inhibited. This observation strongly suggests that the whole mycobacterial cell requires a more complex processing step than that required for handling soluble PPD. Similar observations have been previously reported by ZIEGLER and UNANUE[54] in the *Listeria monocytogenes* system. In this study, lysosomotropic agents were shown to inhibit both degradation of the antigen by murine macrophages and the capacity of these cells to present bacterial epitopes in an antigen-specific binding and proliferation assay. Taken all together, these studies point out a common mode of handling intracellular pathogens by macrophages and B cell lymphomas. A still open question is whether microbial-activated normal B cells can be compared to B cell lymphomas in successfully handling the whole bacteria.

The mechanisms by which B cells process protein antigens and present the resulting fragments to T cells have not been as well explored as with macrophages, but the role of the immunoglobulin receptor in these processes has been recently addressed. ROCK et al.[46] showed that murine 2,4,6-trinitrophenyl (TNP)-specific B cells are 1,000-fold more efficient in presenting TNP-coupled synthetic polymer (Glu-Lys-Phe) to a polymer-specific T cell hybrid, if compared to the presentation of the polymer in absence of TNP, indicating that the presentation of the polymer is enhanced by the uptake of the molecule *via* the TNP-specific immunoglobulin receptor. In the human system, LANZAVECCHIA[26]

similarly showed that tetanus toxoid-specific EBV-B cells were able to present the antigen to specific T cell lines at concentration four orders of magnitude lower than required for presentation by nonspecific B cells or peripheral blood monocytes. The role of the immunoglobulin receptor to concentrate the antigen on specific B cell surface for subsequent internalization, processing and presentation has been also analyzed by MALYNN et al.[33] in a model in which a particulate form of antigen was used. In fact, they showed that murine antigen-primed B cells were able to present sheep red blood cells to specific T cell clones. However, in such an experimental model the possibility that sheep red blood cells could release soluble antigens in culture and/or that macrophages present within the population could process the antigen (which is then taken up and presented by B cells) cannot be excluded. Our experimental model overcame this possible criticism because supernatants from the whole *M. tuberculosis* cells failed to activate T cells, thus excluding the presence of soluble molecules contaminating the whole-H37Rv preparation. Moreover, the use of EBV-B as antigen-presenting cells and the use of T cell clones as responder population allow us to exclude macrophages as responsible for *M. tuberculosis* presentation.

To investigate the role of the immunoglobulin receptors in mycobacterial uptake and T cell activation, PPD-specific and nonspecific EBV-B cell clones were selected by their capacity to produce anti-PPD antibodies[16]. Figure 2 shows the difference between PPD-specific and nonspecific EBV-B cell clones in the presentation of soluble and particulate antigens to T cell clones in an 18-h pulsing experiment. As it can be seen, the level of T cell proliferation induced by PPD-specific EBV-B cells is higher than that observed with nonspecific EBV-B cells at all cell ratios used, both with soluble and particulate forms of antigen.

Although the present and previous reports[6,7,17] have clearly shown that the presentation of the antigen by B cell lymphomas occurs without any apparent need of the surface immunoglobulin specificity for the antigen, the specific interaction between antigen and surface immunoglobulin receptors may be necessary when lower and more physiologic doses of the antigen are used[26,33,46]. The interaction of mycobacterial antigens with specific B cells could serve not

treatment of EBV-B cells	PPD pulsing		whole-H37Rv pulsing	
	I	II	I	II
none	5,515	51,042	8,174	18,961
chloroquine	6.252	41,753	1,282	3,918
	(0)	(18)	(84)	(79)

Chloroquine (10^{-4} M) was added to EBV-B cells ($5x10^5$/ml) during the pulsing period of 18h in the presence of either PPD or whole-H37Rv. At the end of pulsing, EBV-B cells were washed three times, irradiated using a low dose (3,000 rads) and then added to cultures. In the experiment I, the responder cell population consisted of T cell lines, while in the experiment II purified resting T cells were used. All cultures were harvested after 4 days and radioactive thymidine was added in the last 12h of culture. The results are expressed as cpm. The percentage of inhibition of antigen presentation by chloroquine treatment is shown in brackets.

Tab. 2 - Effect of chloroquine treatment on the antigen presentation by EBV-B cells.

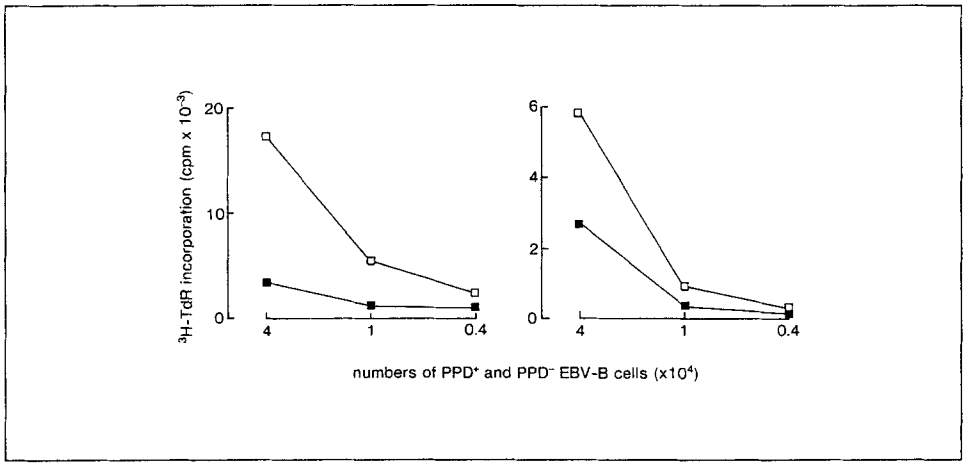

Fig. 2 - Presentation of soluble (*on the left*) and particulate (*on the right*) forms of mycobacterial antigen by PPD-specific and nonspecific EBV-B cell clones. PPD-specific (□) and nonspecific (■) EBV-B cells were pulsed for 18h in the presence of PPD (*on the left*) or whole-H37Rv (*on the right*) antigens. After pulsing, the cells were washed, irradiated (7,500 rads) and added at different numbers to T cell clone DGA1. Cells were cultured for 4 days and radioactive thymidine (^{3}H-TdR) was added 20h before harvesting.

only to focus the antigen or to change the activation state of the B cell, but also to select the antigenic determinants to be presented to T cells. In fact, recent data from Celada's group have shown that β-galactosidase-specific B cells present specific determinants different from those recognized by the immunoglobulin receptors to T cell clones (F. Manca, personal communication). As a consequence of these observations, the role of specific B lymphocytes in the tuberculosis has to be considered, not only in terms of antibody production but also in the context of the antigen presentation and selection of mycobacterial determinants for T cell activation.

2. *Activation and characterization of mycobacterial-specific T lymphocytes*

It has recently become possible to maintain antigen-specific and functionally active T lymphocytes in monoclonal form[36]. This monoclonal approach is providing more information on the T cell subpopulations involved in the microbial infections carried out by intracellular parasites, such as the malaria[49], lepra[42] and *Listeria*[21] ones. T cell clones and lines from tuberculosis patients have been developed in our laboratories for two purposes: *i.* characterization of mycobacterial-specific T cell subpopulations and their biological activities, *ii.* analysis of the antigenic determinants important in the anti-mycobacterial immunity.

Peripheral blood mononuclear cells from both tuberculosis patients and BCG-vaccinated healthy donors were stimulated *in vitro* with distinct antigenic *M. tuberculosis* preparations, and then cloned and propagated as recently described[27]. Autologous EBV-transformed B cells were used as APC as above reported. One advantage of using EBV-B cells as APC is that such cells provide a continuous and unlimited source of APC, thus reducing the number of

autologous mononuclear cells needed for the propagation of antigen-specific TLC. Thirty mycobacterial-reactive proliferating TLC were randomly selected and studied in a more detailed way as far as their antigen specificities were concerned. All tested TLC had the $T3^+$ and $T4^+$ phenotypes, expressed CD25 antigen (IL-2 receptor) and were strongly positive for HLA-DR. Table 3 illustrates their proliferative responses to different microbial antigenic preparations. As it can be seen, all TLC respond to an optimal dose of *M. tuberculosis* extract from the H37Rv strain or to PPD. All these TLC were also cross-reactive to *M. bovis*, but failed to recognize other mycobacterial species such as *M. gordonae* or *M. avium*. None of the TLC showed proliferative responses in the presence of APC and tetanus toxoid (TT) or *Candida albicans* polysaccharide extract (MPPS), which were tested as non-mycobacterial control antigens. All TLC tested were found to be HLA-DR restricted in their response, as shown by inhibition studies.

The TLC reactivity was also evaluated in terms of γ-IFN production. The analysis of this lymphokine has been chosen since it is widely accepted that γ-IFN plays a major role in the macrophage activation and hence in anti-mycobacterial immunity[18,38]. The production of γ-IFN after stimulation with human recombinant IL-2 (rIL-2) of TLC was also investigated: supernatants from activated TLC in the presence or absence of rIL-2 were collected at 24 and 48h, and their γ-IFN activity was biologically tested as previously reported[44]. Table 4 shows γ-IFN production in culture supernatants from three mycobacterial-specific and PPD-stimulated TLC in the presence or absence of rIL-2. Mycobacterial-specific human T cell clones have been recently shown to release other molecules with macrophage-activating properties different from γ-IFN[2,34], indicating the complexity of the T cell-macrophage interaction in the expression phase of the immune response to *M. tuberculosis*.

Although it is well demonstrated that protective immunity against intracellular bacteria including *M. tuberculosis* is mediated by specific helper T cells that activate macrophages, there are some recent evidences that specific cytotoxic T

antigens	Ew4/1	Ew4/7	Ew4/10	Gw8/9	Gw8/16	DG2/2	DG2/5
–	242	207	182	161	279	945	430
PPD	8,939	6,231	15,728	959	18,582	5,494	14,389
H37Rv	9,307	7,656	7,071	19,015	28,584	nd	nd
M. bovis	10,038	5,737	9,143	5,723	15,067	nd	nd
M. gordonae	471	284	240	182	340	nd	nd
M. avium	268	380	164	398	254	nd	nd
MPPS	266	231	254	184	263	928	500
TT	217	258	278	198	292	980	370

T cell clones (10^4 cells/well) were cultured for 4 days with irradiated autologous PBMC (10^5 cells/well). The clones DG2/2 and DG2/5 were cultured with irradiated EBV-B cells (10^4 cells/well) as APC. The antigen preparations were added at the beginning of the cultures, whereas radioactive thymidine (0.5 μCi/well) was added 12h before the end of cultures. The DNA synthesis was expressed as cpm and the standard deviation was always less than 10%.

Tab. 3 - Proliferative response of mycobacterial-specific T cell clones.

TLC	h	culture conditions					
		APC		APC (PPD)		APC (PPD) + rIL-2	
		^{3}H-TdR	IFN	^{3}H-TdR	IFN	^{3}H-TdR	IFN
DG2/2	24	2,199	<3	8,318	10	61,388	30
	48	2,475	<3	16,192	30	75,936	30
DG2/5	24	2,312	<3	6,501	30	30,934	100
	48	2,474	<3	16,518	30	41,291	30
DG2/8	24	nd	<3	nd	10	21,626	30
	48	nd	<3	nd	10	28,993	20

TLC were cultured for 3 days in the presence of APC alone, APC plus PPD or APC plus PPD and rIL-2 (10 U/ml). The 24-h culture supernatants were titrated for γ-IFN activity (30). The radioactive thymidine (^{3}H-TdR) was added 12h before harvesting and the results are reported as cpm.

Tab. 4 - DNA synthesis and IFN production in TLC cultures stimulated with PPD and IL-2.

lymphocytes are also involved in the protection against mycobacterial infections[41]. Moreover, human cytotoxic T cells from BCG-vaccinated volunteers able to kill more efficiently than allogeneic ones autologous antigen-labelled target cells have been reported[19]. This suggests the possibility that cytotoxic T cells may play a protective role in tuberculosis. The generation of murine cytotoxic T cell clones specific for *Listeria monocytogenes* has been recently described[23] and their putative role in the protection has been suggested. In our laboratories we are investigating this aspect by developing $CD8^+$ cytotoxic T cell clones and using EBV-B as target cells.

The T cell cloning approach is also useful to answer the question of the antigenic determinants of *M. tuberculosis* important in anti-mycobacterial immunity. This key point has been approached biochemically by fractionation and partial purification of mycobacterial components for more than 50 years, but no conclusive evidences have been reached at the level of protein determinants involved in T cell activation. In fact, both the antigenic preparations used for immunodiagnosis (PPD) and vaccination (BCG) of tuberculosis are not judged efficient by clinical and epidemiological studies. A second generation of defined mycobacterial antigens useful in immunodiagnosis and prophylaxis may be prepared using the DNA recombinant technology. Recently, YOUNG et al.[53] developed a λ-gT11 recombinant DNA library of *M. tuberculosis*. Recombinant genomic DNA libraries can be screened with antibody probes[52,53] to point out antigens produced by specific recombinant clones. The feasibility of this approach has been recently reported by THOLE et al.[50], who have isolated a 64-kD protein by screening a *M. bovis*-BCG library. Kaufmann's group[14] showed that such a recombinant protein specifically stimulates human T4 clones reactive to mycobacterial antigens. In our laboratories, relevant recombinant mycobacterial determinants are identified by screening the *M. tuberculosis* DNA library using human monoclonal antibodies[16] and sera from tuberculosis patients. Such recombinant antigens are then tested with our panel of TLC in a proliferative assay. Work is in progress to establish a relationship between recombinant products and T cell function.

The determination of the antigenic structure of *M. tuberculosis* will allow to investigate in more details the induction of the immune response leading not only to the protection but also to the host's tissue damage. The negative effects of the immune response to *M. tuberculosis* may be due to both the T and the B cell mechanisms. The production of IL-2 and IFN by mycobacterial-specific T lymphocytes can induce: *i.* the activation of macrophages and then the release of proteolytic enzymes (classical delayed-type hypersensitivity), and *ii.* the development of autoreactive T cell clones (autoimmunity). In fact, γ-IFN is known to increase the expression of MHC class II molecules on macrophages, B cells and other non-immunologically relevant cells[1,32]. Moreover, recent observations suggest that autoreactive T cell clones may locally arise where IFN is released[32] and frequently develop in PBMC cultures stimulated with PPD (unpublished observations) and other microbial antigens[15]. The negative effects of B cell reactivity can be reported to the production of anti-mycobacterial antibodies. In turn, mycobacterial-specific B cells and antibodies may be able to induce the production of auto-anti-idiotypic antibodies. In fact, an anti-idiotypic response has been reported to arise during a murine mycobacterial infection and to suppress the mycobacterial-specific T cell reactivity[11]. Therefore, the identification of antigenic determinants responsible for the activation of T and B cells which lack a protective role but possess negative effects is a goal of the paramount importance in the preparation of a second generation vaccine for the control of tuberculosis.

3. *Cellular and molecular mechanisms regulating the immune response to M. tuberculosis*

There is a large number of data describing the down regulation of mycobacterial-specific immune response in experimental animals. Several possibilities, including defective macrophage function, absence of reactive T cells from the circulation and active suppression carried out by distinct cell populations have been suggested to explain the regulation of DTH response to mycobacterial antigens. However, the mechanism of such down regulation and its cellular target is still unclear. Furthermore, no information is available on the regulation of anti-mycobacterial immunity at the clonal level in humans.

In our laboratories, we have previously described the activation of T suppressor cells by the *Candida albicans* polysaccharide MPPS[45] and, more recently, the characterization of an antigen nonspecific inhibitory molecule(s) (nsINH) released *in vitro* by human $CD8^+$ T cells specifically activated with recall microbial antigens such as MPPS and PPD[28-31]. The possibility that proteins present in the PPD preparation contain determinants able to activate distinct T cell subsets, including T suppressor cells, has been reported in other models by showing the presence of both suppressor and helper epitopes in the same molecule[9]. Molecules with antigen nonspecific and genetic unrestricted inhibitory activity have been described as the final component of the complex T suppressor cell circuit which regulates contact hypersensitivity to haptens in the mouse (for a review, see ASHERSON et al.[3]). In the present model, nsINH produced by PPD-activated PBMC has been reported to inhibit both proliferation and natural killer cell induction by interfering with the lymphokine cascade at more than one level. In fact, the release of IL-1 production by PPD-activated monocytes[31] and the production of IL-2 and γ-interferon by MPPS- or PPD-

activated T cells as well as the expression of IL-2 receptor[28] are inhibited by the addition of nsINH at the beginning of the culture.

The mechanism of action of such nsINH has been then investigated by using the TLC panel; nsINH was added at the beginning of the cultures containing mycobacterial-specific TLC, irradiated APC and antigen (*M. bovis* or H37Rv or PPD). Table 5 shows that nsINH produced by MPPS- or PPD-activated PBMC constantly inhibits the mycobacterial-specific TLC proliferation, thus confirming previous results on the antigen nonspecific activity of the nsINH. Preliminary evidences suggest that the T cell is the target of nsINH activity. In fact, the inhibition of TLC proliferation is also observed when nsINH is preincubated with T cell clones, then removed, and TLC are cultured in fresh medium with APC and antigen.

Other lymphokines with regulatory functions such as lymphotoxin (LT)[47] and tumor necrosis factor (TNF)[43] have been described and recently cloned. To exclude the possibility that nsINH activity is LT or TNF in nature, we have used anti-LT and anti-TNF monoclonal antibodies to neutralize the contaminating putative LT and TNF activity on nsINH-containing supernatants. Table 6 shows that anti-TNF antibodies fail to block the inhibitory activity of nsINH, whereas they are fully able to remove TNF activity. Similar findings are observed with anti-LT antibodies. These results suggest that nsINH is a regulatory lymphokine distinct from LT and TNF.

The findings here reported on the inhibitory effect of nsINH on mycobacterial-specific TLC open the question about the biological role of nsINH in human tuberculosis. It is well known that the advanced disseminated form of pulmonary tuberculosis is accompanied by a state of immunological unresponsiveness to several antigens, as judged *in vivo* by skin tests[4,40]. Furthermore, peripheral blood mononuclear cells from such patients fail to proliferate and to release IL-2 and γ-IFN when stimulated *in vitro* with PPD[13,40]. However, they

TLC	antigen	addition to culture of		
		medium	$nsINH_{MPPS}$	$nsINH_{PPD}$
Gw8/9	H37Rv	3,291	1,500 (49)	396 (83)
Ew4/1	H37Rv	4,461	2,486 (44)	nd
Ew4/10	H37Rv	2,753	1,827 (33)	nd
Gw8/16	*M. bovis*	3,976	2,024 (49)	733 (81)
DG2/2	PPD	13,594	nd	9,346 (31)
DG2/2*	PPD	6,418	nd	2,233 (46)
DG2/5	PPD	2,904	nd	2,147 (26)
DG2/5*	PPD	6,340	nd	4,151 (35)

T cell clones (10^4 cells/well) were cultured with irradiated PBMC (10^5 cells/well) or EBV-B cells (10^4 cells/well)* and different mycobacterial antigen preparations in the absence or presence of 50% $nsINH_{MPPS}$ or $nsINH_{PPD}$. The radioactive thymidine was added 12h before harvesting. The results are reported as cpm. Background proliferation (T cell clones with APC without antigen) was less than 10%. Numbers in brackets indicate the percentage of inhibition.

Tab. 5 - Effect of nsINH on mycobacterial-specific T cell clones.

nsINH	monoclonal antibodies	TNF	cpm (% inhibition)
–	–	–	78,659
+	–	–	51,534 (34)
+	anti-LT	–	51,249 (35)
+	anti-TNF	–	54,071 (31)
–	–	+	42,802 (45)
–	anti-LT	+	45,277 (42)
–	anti-TNF	+	73,630 (6)

Mycobacterial-specific T cell clones (Ew4/7) were cultured with PBMC and H37Rv; nsINH containing supernatants pretreated (6h at 4 °C) with monoclonal antibodies anti-lymphotoxin (anti-LT) or anti-tumor necrosis factor (anti-TNF) were added at the beginning of the culture. TNF (10^4 U) was used in the control experiment instead of nsINH. Data are reported as cpm and the percentage of inhibition is shown in brackets.

Tab. 6 - Effect of anti-LT and anti-TNF monoclonal antibodies on nsINH activity.

can express IL-2 receptor and the addition in culture of recombinant IL-2 enhances both cell proliferation and IFN production[13, 50]. Similar observations are found in mice injected with high doses of *M. bovis*, strain BCG. Their lymphocytes fail to react to PPD, but this unresponsiveness can be reversed both *in vitro* and *in vivo* by the administration of exogenous IL-2[8] or feeding the infected mice on a diet supplemented with vitamin A acetate[12], treatment which is known to increase the IL-2 production. It is interesting to note that spleen cells from BCG-infected mice release a nsINH in culture which blocks both cell proliferation and IL-2 production[10]. These studies in both the human and murine models might suggest a role of nsINH in the control of the immune response to *Mycobacteria*, although a cause-effect relationship between nsINH and mycobacterial immunological unresponsiveness is still lacking.

In chronic infections, low T cell activation and lymphokine production may lead to a situation in which microorganisms persist in insufficiently activated macrophages. Under these circumstances, the inhibition of nsINH production or the increase of IL-2 may reach helpful effects. Immunotherapy with T cell clones and rIL-2 in mycobacterial[8, 41] and listeriosis[22] infections has provided preliminary evidence that such approach could be highly promising to activate antibacterial mechanisms in the host.

CONCLUSIONS

Figure 3 shows a complex scenario with cells and molecules involved in the immune response to *M. tuberculosis*, as it is possible to draw with the data presented in this review. As it can be seen, the antigenic complexity of *M. tuberculosis* allows the activation of both B and T cells, the latter displaying distinct phenotypes and functions, i.e., helper, cytotoxic and suppressor. The helper cells mediate antitubercular immunity both activating macrophages through the release of γ-IFN and MAF and inducing the differentiation and proliferation of mycobacterial-specific cytotoxic T cells through the production of IL-2. Other mycobacterial determinants may activate T suppressor cells

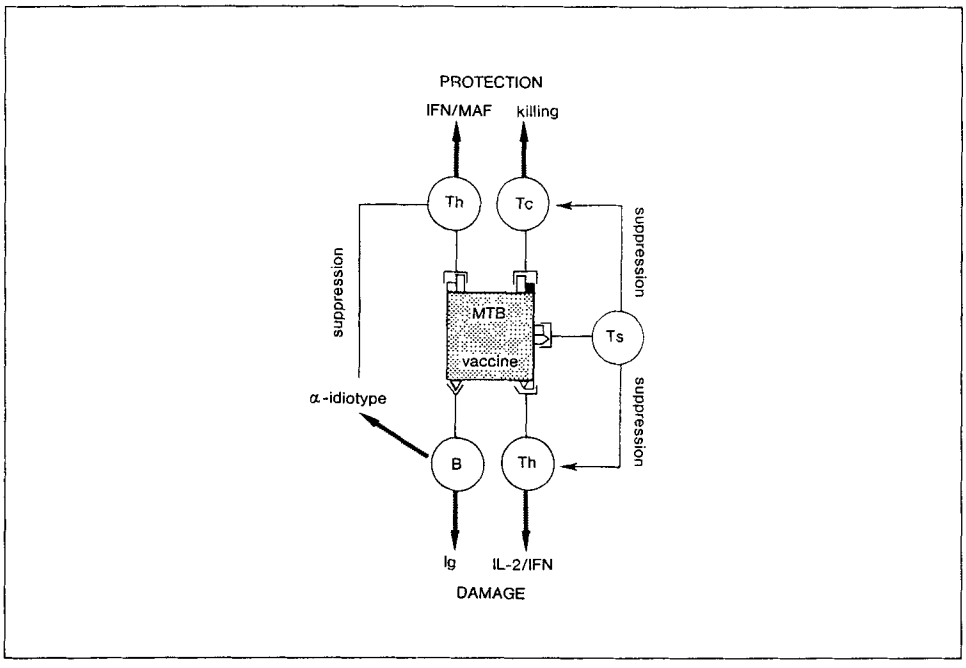

Fig. 3 - Diagram illustrating the complexity of the immune response to *M. tuberculosis* (MTB). The focus is on the distinct helper and suppressor epitopes present on mycobacterial proteins and on the subsequent activation of T helper and suppressor cells. Protection and/or tissue damages are the final effects of the immune response to *M. tuberculosis*.

which regulate the undergoing immune response by the release of nsINH, a molecule functionally similar to other lymphokines for its antigen nonspecific and genetically unrestricted mode of action. The role of B lymphocytes in antitubercular immunity may be reported to the selection of mycobacterial determinants presented to T cells. However, as it can be seen from the lower part of fig. 3, tissue damages can occur during the immune response to *Mycobacteria*, and both T and B cell mechanisms are at the basis of such harmful effects. The identification by the recombinant DNA technology of antigenic determinants specific for the activation of T cells with protective function and the possibility to abrogate the immunological unresponsiveness by the administration of exogenous IL-2 are the main directions of research for immunologists in the field of tuberculosis.

SUMMARY

Tuberculosis is still one of the major health problems in almost all over the world. Thus, new directions in basic and applied research on tuberculosis are under investigation. In this review we have provided recent data obtained in our laboratories on three main aspects of the immunology of tuberculosis, namely: *i.* the role of B lymphocytes in the processing and presentation of *Mycobacterium tuberculosis* antigens to T cells; *ii.* the activation and characterization of mycobacterial-specific T cell clones; *iii.* the T cell regulation of the immune response to *M. tuberculosis*. The analysis of the antigenic determinants of *M. tuberculosis* relevant in the antimycobacterial immunity is the major goal of the WHO programme on the immunology of tuberculosis. In

fact, the attempt to develop a second generation vaccine against this microorganism is now possible by analyzing recombinant genomic DNA libraries of *M. tuberculosis* with monoclonal antibodies and T cell clones. In the near future, the identification of epitopes recognized by mycobacterial-specific T cells with helper, cytotoxic and suppressor functions will allow the preparation of recombinant and synthetic vaccines effective in the control of this disease.

ACKNOWLEDGEMENTS

The authors wish to thank C. Garzelli for providing EBV-B transformed B cells and anti-PPD monoclonal antibodies, J. Ivanyi for the soluble preparation of *M. tuberculosis* antigen, O. Pugliese for the immunofluorescence determinations, P. Pizzoli for γ-IFN titration, C. De Martino for electron microscopy studies and M. Gobbi for reviewing the manuscript. We are also indebted to D. Guerritore for his constant encouragement.

REFERENCES

1. AMEGLIO F., CAPOBIANCHI M. R., DOLEI A., TOSI R.: Differential effects of gamma interferon on expression of HLA class II molecules controlled by the DR and DC loci · Infect. Immun. *42*, 122, 1983.
2. ANDREW P. W., REES A. D. M., SCOGING A., DOBSON N., MATTHEWS R., WHITTALL J. T., COATES A. R. M., LOWRIE D. B.: Secretion of macrophage-activating factor distinct from interferon gamma by human T cell clones · Europ. J. Immunol. *14*, 962, 1984.
3. ASHERSON G. L., COLIZZI V., ZEMBALA M.: An overview of T-suppressor cell circuits · Ann. Rev. Immunol. *4*, 37, 1986.
4. CHAPARAS S. D.: Immunity in tuberculosis · Bull. Wld Hlth Org. *60*, 447, 1982.
5. CHEN-WOAN M., MACGREGOR D. D.: The mediators of acquired resistance to *Listeria monocytogenes* are contained within a population of cytotoxic T cells · Cell. Immunol. *87*, 538, 1984.
6. CHESNUT R. W., COLON S. M., GREY H. M.: Antigen presentation by normal B cells, B cell tumors, and macrophages: functional and biochemical comparison · J. Immunol. *128*, 1764, 1982.
7. CHU E., UMETSU D., LAREAU M., SCHNEEBERGER E., GEHA R. S.: Analysis of antigen uptake and presentation by Epstein-Barr virus-transformed human lymphoblastoid B cells · Europ. J. Immunol. *14*, 291, 1984.
8. COLIZZI V.: *In vivo* and *in vitro* administration of interleukin-2-containing preparation reverses T cell unresponsiveness in *M. bovis* BCG-infected mice · Infect. Immun. *45*, 25, 1984.
9. COLIZZI V., ADORINI L., DORIA G.: Immunoregulation of lysozyme-specific suppression. I. Induction and suppression of delayed-type hypersensitivity to hen egg-white lysozyme · Europ. J. Immunol. *14*, 820, 1984.
10. COLIZZI V., ASHERSON G. L., MALKOVSKY M., GARREAU F., FERLUGA J.: Suppressor cells induced by BCG release nonspecific factors *in vitro* which inhibit DNA synthesis and IL-2 production · Immunology *51*, 655, 1984.
11. COLIZZI V., GIUNTINI M., GARZELLI C., CAMPA M., FALCONE G.: Auto-antiidiotypic antibodies inhibit T cell-mediated hypersensitivity in BCG-infected mice · Cell. Immunol. *80*, 205, 1983.
12. COLIZZI V., MALKOVSKY M.: Augmentation of interleukin-2 production and delayed hypersensitivity in mice infected with *Mycobacterium bovis* and fed a diet supplemented with vitamin A acetate · Infect. Immun. *48*, 581, 1985.
13. DEL GALLO F., LOMBARDI G., VISMARA D., PICCOLELLA E., PIZZOLI P., BUFARDECI E., DOLEI A., ARIENZO F., GIDARO M., DI GIACOMO P., COLIZZI V.: Effect of recombinant IL-2 on mycobacterial T cell unresponsiveness in advanced disseminated tuberculosis · Immunology. (Submitted for publication).
14. EMMERICH F., THOLE J., VAN EMBDEN J., KAUFMANN H. E.: A recombinant 64 kD protein of *Mycobacterium bovis* BCG specifically stimulates human T4 clones reactive to mycobacterial antigens · J. exp. Med. *163*, 1024, 1986.
15. FAHERTY D. A., JOHNSON D. R., ZAUDERER M.: Origin and specificity of autoreactive T cells in antigen-induced populations · J. exp. Med. *161*, 1293, 1985.

16. GARZELLI C., PUGLISI C., FALCONE G.: Human monoclonal antibody to purified protein derivative produced by hybrids constructed with Epstein-Barr virus-transformed B lymphocytes and mouse myeloma cells - Europ. J. Immunol. *16*, 584, 1986.

17. GREY H. M., COLON S. M., CHESNUT R. W.: Requirements for the processing of antigen by antigen-presenting B cells. II. Biochemical comparison of the fate of antigen in B cell tumors and macrophages - J. Immunol. *129*, 2389, 1982.

18. HAHN H., KAUFMANN S. H. E.: The role of cell-mediated immunity in bacterial infections - Rev. infect. Dis. *3*, 1221, 1981.

19. HANK J. A., SONDEL P. M.: Soluble bacterial antigen induces specific helper and cytotoxic responses by human lymphocytes *in vitro* - J. Immunol. *128*, 2734, 1982.

20. KAPSENBERG M. L., TEUNISSEN M. B. M., STIEKEMA F. E. M., KEIZER H. G.: Antigen-presenting cells of dendritic cells and macrophages in proliferative T cell response to soluble and particulate antigens - Europ. J. Immunol. *16*, 345, 1986.

21. KAUFMANN S. H. E.: Effective antibacterial protection induced by a *Listeria monocytogenes*-specific T cell clone and its lymphokines - Infect. Immun. *39*, 1265, 1983.

22. KAUFMANN S. H. E.: T cell clones in the study of acquired resistance to facultative intracellular bacteria. In: VON BOEHMER H., HAAS W. (Eds): T cell clones. Elsevier Sci. Publ., Amsterdam, 1985.

23. KAUFMANN S. H. E., HUG E., DE LIBERO G.: *Listeria monocytogenes*-reactive T lymphocyte clones with cytolytic activity against infected target cells - J. exp. Med. (In press).

24. KAUFMANN S. H. E., SIMON M. M., HAHN H.: Regulatory interactions between macrophages and T cell subsets in *Listeria monocytogenes*-specific T cell activation - Infect. Immun. *38*, 907, 1982.

25. KAYE P. M., CHAIN B. M., FELDMANN M.: Nonphagocytic dendritic cells are effective accessory cells for anti-mycobacterial responses *in vitro* - J. Immunol. *134*, 1930, 1985.

26. LANZAVECCHIA A.: Antigen-specific interaction between T and B cells - Nature (Lond.) *314*, 537, 1985.

27. LOMBARDI G., DEL GALLO F., VISMARA D., PICCOLELLA E., DE MARTINO C., GARZELLI C., PUGLISI C., COLIZZI V.: Epstein-Barr virus-transformed B cells process and present *Mycobacterium tuberculosis* particulate antigens to T cells - Cell. Immunol. (In press).

28. LOMBARDI G., DI MASSIMO A. M., DEL GALLO F., VISMARA D., PICCOLELLA E., PUGLIESE O., COLIZZI V.: Mechanism of action of an antigen nonspecific inhibitory factor produced by human T cells stimulated by MPPS and PPD - Cell. Immunol. *98*, 434, 1986.

29. LOMBARDI G., PICCOLELLA E., VISMARA D., COLIZZI V., ASHERSON G. L.: *Candida albicans* polysaccharide extract (MPPS) and PPD stimulate the production of IL-1 and lymphocyte proliferation - Clin. exp. Immunol. *58*, 581, 1984.

30. LOMBARDI G., PICCOLELLA E., VISMARA D., COLIZZI V., ZEMBALA M.: Monocyte subsets in the production of inhibitory factor by *Candida albicans*-activated human T cells - Immunology *56*, 373, 1985.

31. LOMBARDI G., VISMARA D., PICCOLELLA E., COLIZZI V., ASHERSON G. L.: A non-specific inhibitor produced by *Candida albicans*-activated T cells impairs cell proliferation by inhibiting interleukin-1 production - Clin. exp. Immunol. *60*, 303, 1985.

32. LONDEI M., LAMB J. R., BOTTAZZO G. F., FELDMANN M.: Epithelial cells expressing aberrant MHC class II determinants can present antigen to cloned human T cells - Nature (Lond.) *312*, 639, 1985.

33. MALYNN B. A., ROMEO D. T., WORTIS H. H.: Antigen-specific B cells efficiently present low doses of antigen for induction of T cell proliferation - J. Immunol. *135*, 980, 1985.

34. MATTHEWS R., SCOGING A., REES A. D. M.: Mycobacterial antigen-specific human T cell clones secreting macrophage-activating factors - Immunology *54*, 17, 1985.

35. MINISTERO DELLA SANITÀ: Inchiesta epidemiologica sulla TBC - Circolare n. 88, 1983.

36. MÖLLER G. (Ed.): T cell clones - Immunol. Rev. *54*, 1, 1981.

37. MORETTA A., PANTALEO G., LOPEZ-BOTET M., MINGARI M. C., MORETTA L.: Anticlonotypic monoclonal antibodies induce proliferation of clonotype-positive T cells in peripheral blood human T lymphocytes - J. exp. Med. *162*, 1393, 1985.

38. NATHAN C. F., MURRAY H. W., WIEBE M. W., RUBIN B. Y.: Identification of interferon as the lymphokine that activates human macrophages oxidative metabolism and antimicrobial activity · J. exp. Med. *158*, 2159, 1983.

39. NATHAN C. F., NAKAGAWARA A.: Role of reactive oxygen intermediates in macrophage killing of intracellular pathogens: a review. In: MIZUNO D., COHN Z. A., TAKEYA K., ISHIDA N. (Eds): Self-defence mechanism: role of macrophages. University of Tokyo Press, Tokyo, 1982; p. 279.

40. ONWUBALILI J. K., SCOTT G. M., ROBINSON J. A.: Deficient immune interferon production in tuberculosis · Clin. exp. Immunol. *59*, 405, 1985.

41. ORME I. M., COLLINS F. M.: Protection against *M. tuberculosis* infection by adoptive immunotherapy · J. exp. Med. *158*, 74, 1983.

42. OTTENHOFF T. H. M., KLATSER P. R., IVANYI J., ELFERINK D. G., DE WIT M. Y. L., DE VRIES R. R. P.: *Mycobacterium leprae*-specific protein antigens defined by cloned human helper T cells · Nature (Lond.) *319*, 427, 1986.

43. PENNICA D., NEDWIN G. E., HAYFLICK J. S., SEEBURG P. H., DERYNCK R., PALLADINO M. A., KOHR W. J., AGGARWAL B. B., GOEDDEL D. V.: Human tumor necrosis factor: precursor, structure, expression and homology to lymphotoxin · Nature (Lond.) *312*, 724, 1984.

44. PICCOLELLA E., DOLEI A., LOMBARDI G., VISMARA D., PIZZOLI P., COLIZZI V., DIANZANI F.: Regulation of immune interferon production during the response to soluble microbial antigens · Clin. exp. Immunol. *65*, 191, 1986.

45. PICCOLELLA E., LOMBARDI G., MORELLI R.: Generation of suppressor cells in the response of human lymphocytes to a polysaccharide purified from *Candida albicans* · J. Immunol. *126*, 2151, 1981.

46. ROCK K. L., BENACERRAF B., ABBAS A. K.: Antigen presentation by hapten-specific B lymphocytes. I. Role of surface Ig receptors · J. exp. Med. *160*, 1102, 1984.

47. RUDDLE N. H.: Lymphotoxin redux · Immunol. Today *6*, 156, 1985.

48. SCHWARTZ R. H.: T lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex · Ann. Rev. Immunol. *3*, 237, 1985.

49. SINIGAGLIA F., PINK J. R. L.: Human T lymphocyte clones specific for malaria (*Plasmodium falciparum*) antigens · EMBO J. *4*, 3819, 1985.

50. THOLE J. E. R., DAUWERSE U. G., GROOTHUIS D. G., SCHOULS L. M., VAN EMBDEN J. D. A.: Cloning of *Mycobacterium bovis* BCG DNA and expression of antigens in *Escherichia coli* · Infect. Immun. *50*, 800, 1985.

51. VISMARA D., LOMBARDI G., PICCOLELLA E., COLIZZI V.: Dissociation between IL-1 and IL-2 production in proliferative response to microbial antigens: restorative effect of exogenous IL-2 · Infect. Immun. *49*, 298, 1985.

52. WHO WORKSHOP: Characterization of antigens recognized by *Mycobacterium*-specific monoclonal antibodies · Infect. Immun. *51*, 718, 1986.

53. YOUNG R. A., BLOOM B. R., GROSSKINSKY C. M., IVANYI J., THOMAS D., DAVIES R. W.: Dissection of *Mycobacterium tuberculosis* antigens using recombinant DNA · Proc. nat. Acad. Sci. (Wash.) *82*, 2583, 1985.

54. ZIEGLER H. K., UNANUE E. R.: Decrease in macrophage antigen catabolism caused by ammonia and chloroquine is associated with inhibition of antigen presentation to T cells · Proc. nat. Acad. Sci. (Wash.) *79*, 175, 1982.

Requests for reprints should be addressed to:

VITTORIO COLIZZI
Istituto di Microbiologia
Università degli Studi di Pisa
Via S. Zeno 39, 56100 Pisa - Italia