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MHC-Restricted Depletion of Human Myelin Basic Protein–Reactive T Cells by T Cell Vaccination

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Activated autoreactive T cells are potentially pathogenic and regulated by clonotypic networks. Experimental autoimmune diseases can be treated by inoculation with autoreactive T cells (T cell vaccination). In the present study, patients with multiple sclerosis were inoculated with irradiated myelin basic protein (MBP)–reactive T cells. T cell responses to the inoculates were induced to deplete circulating MBP-reactive T cells in the recipients. Regulatory T cell lines isolated from the recipients inhibited T cells used for vaccination. The cytotoxicity of the $CD8^+$ T cell lines was restricted by major histocompatibility antigens. Thus, clonotypic interactions regulating autoreactive T cells in humans can be induced by T cell vaccination.

Circulating autoreactive T cells can be isolated from the blood of normal individuals (*1*). The activation of autoreactive T cells may lead to their pathogenicity in the induction of autoimmune diseases (*2*, *3*). For example, in multiple sclerosis (MS), a chronic inflammatory disease of the central nervous system (CNS) characterized by infiltration of T cells and macrophages, an increased frequency of activated as opposed to resting MBP-reactive T cells is found in patients with active disease (*4*). These autoreactive T cells are probably clonally expanded and accumulate in the MS lesions (*5*). The mechanism by which MBP-autoreactive T cells are regulated in the disease is unknown.

Experimental autoimmune encephalomyelitis (EAE), a paralytic disease of the CNS resembling MS, is an animal model to investigate the regulation of autoreactive T cells (*6*). Small numbers of activated, but not resting, MBP-reactive T cells can rapidly induce the disease on adoptive transfer into naïve animals (*7*). These pathogenic T cells can also be isolated from unprimed rats to mediate lethal EAE (*8*). The MBP-reactive T cells are part of the normal T cell repertoire and may naturally be regulated by clonotypic networks (*9*, *10*). The regulation is postulated to involve recognition of T cell receptor (TCR) V_β chains in the context of major histocompatibility complex (MHC) molecules (*10*, *11*). This concept has led to the paradigm of T cell vaccination, in which attenuated MBP-autoreactive T cells are used to up-regulate clonotypic regulatory networks to prevent and treat EAE (*12*).

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We investigated the nature of T cell–specific clonotypic responses to autologous MBP-autoreactive T cells in vivo and whether the responses are effective in depleting circulating MBP-reactive T cells. These experiments were carried out in patients with MS because regulation of MBP-reactive T cells has potential therapeutic consequences in the disease.

Six patients with clinically definite MS participated in this phase 1 trial. Three patients were diagnosed as having relapsing-remitting MS, and three other patients had primary and chronic progressive MS. The MBP-reactive T cell lines were first generated from subjects and then cloned by limiting dilution (*13*). Clinical data of the patients and the fine specificity of the T cell clones used as inoculates are shown in Table 1. In agreement with previous studies (*14*, *15*), this panel of T cell clones reacted to at least four different peptides, with a predominant reactivity pattern to peptide 84–102 and peptide 143–168. Thus, selection of T cell clones for inoculation was based on their reactivity to a predominant peptide, which varied between subjects. For example, three T cell clones reactive to peptide 84–102 were selected for subject GE, since all the clones isolated from this subject responded exclusively to this peptide. Thus, in each case at least two T cell clones reactive either to a predominant MBP peptide or to different epitopes (for example, the clones from BC) were pooled as a vaccine. T cell clones were activated with MBP-pulsed antigen-presenting cells (APCs) 4 days before injection (*16*). A total of three inoculations were given to each recipient by injection of a pool of two or three irradiated T cell clones (10^7 to 1.5×10^7 cells per clone) subcutaneously (*16*).

T cell responses to the inoculates were examined and compared with nonspecific phytohemagglutinin (PHA)-induced autologous T cell blasts prepared concurrently (*17*). All six subjects developed a substantial proliferative response to the inoculates after the second inoculation. The responses were accompanied by a marginal reactivity to PHA-induced T cell blasts (Fig. 1). We examined the frequency of MBP-reactive T cells before and after each inoculation of irradiated MBP-reactive T cell clones (*17*). This revealed a progressive decline of circulating MBP-reactive T cells, most notably after the second inoculation. The decrease in the frequency of MBP-reactive T cells correlated reciprocally with the responses to the inoculates (Fig. 1). The frequency fell below the detectable limit of our assay in five of the six recipients by the end of the trial. MBP-reactive T cells in subject HM could still be detected after the third inoculation, but at a frequency (1.1×10^{-7}) that was only 20% of the preinocu-

lation value. In contrast, the frequency of tetanus toxoid (TT)–reactive T cells remained unchanged in all recipients, as did the frequency of MBP-reactive T cells in two nonrecipient subjects, suggesting a specific depletion of MBP-reactive T cells (Fig. 2). Unlike the T cell responses, antibody reactivity to the inoculates could be detected only in one out of six subjects. Staining of the inoculates with patient serum was analyzed by flow cytometry. The serum samples of a subject obtained after two inoculations reacted with 33 ± 3.2% of the inoculates (as against a background staining of 1.2%), as compared with a baseline value of 3 ± 0.2%. The same samples did not react with autologous PHA-induced T cell blasts (<3 ± 0.3%).

We then isolated responding T cells that could specifically recognize the inoculates. Whole mononuclear cells (WMNC) from inoculated subjects were stimulated with irradiated inoculates to establish cell lines (*18*). Responding cell lines were selected on the basis of specific inhibition (>60%) of the proliferation of the inoculates in response to MBP (*18*). Specific T cells capable of suppressing the proliferation of inoculates were detectable in all three recipients tested, with estimated frequencies of 0.2×10^{-6} (BC), 2.3×10^{-6} (CW), and 5.2×10^{-6} (GE). Disease-matched subjects not inoculated with MBP-reactive T cells were also examined, but in these instances the responding T cells were not observed (Fig. 2). Twenty-four cell lines were selected from two recipients, CW and GE. They inhibited the proliferation of the autologous inoculates by 68 to 98% in three separate experiments. All cell lines expressed the TCRαβ. Twenty-two T cell lines were $CD8^+$ and two were $CD4^+$.

We further examined whether this inhibition required cell contact or whether it was mediated by factors secreted by the T cells. Inhibition of T cell proliferation by the responding T cell lines required T-T cell contact and was not cytokine mediated (*19*). The inhibitory T cell lines were assessed for their functional properties and specificities. Both $CD4^+$ (CW2F3) and $CD8^+$ (CW1G9, GE1B3, and GE1D6) T cell lines were specific inhibitors of the inoculates (Fig. 3A). The T cell lines but not the TT-reactive clones were stimulated specifically by the inoculates (Fig. 3B). All three $CD8^+$ lines lysed the inoculates in a standard 4-hour chromium release assay (Fig. 4A), and this antigen-specific cytotoxicity could be blocked by a monoclonal antibody to MHC class I molecules (W6/32) but not by an antibody to MHC class II products (HB55) (Fig. 4B). Similar results were obtained from seven other $CD8^+$ cell lines.

Thus, these regulatory T cell lines may be termed anti-clonotypic T cells for their specific reactivity to a clonotypic structure of the inoculates (*20, 21*). Our data suggest that the anti-clonotypic T cells could be induced or enhanced by the inoculation and are responsible for depleting circulating MBP-reactive T cells by MHC class I–restricted cytolytic activity. The anti-clonotypic T cells demonstrated here share many functional features similar to those of the anti-clonotypic T cells isolated from vaccinated rats and that are capable of conferring protection against EAE to naïve rats (*11, 21*). However, it should be noted that the

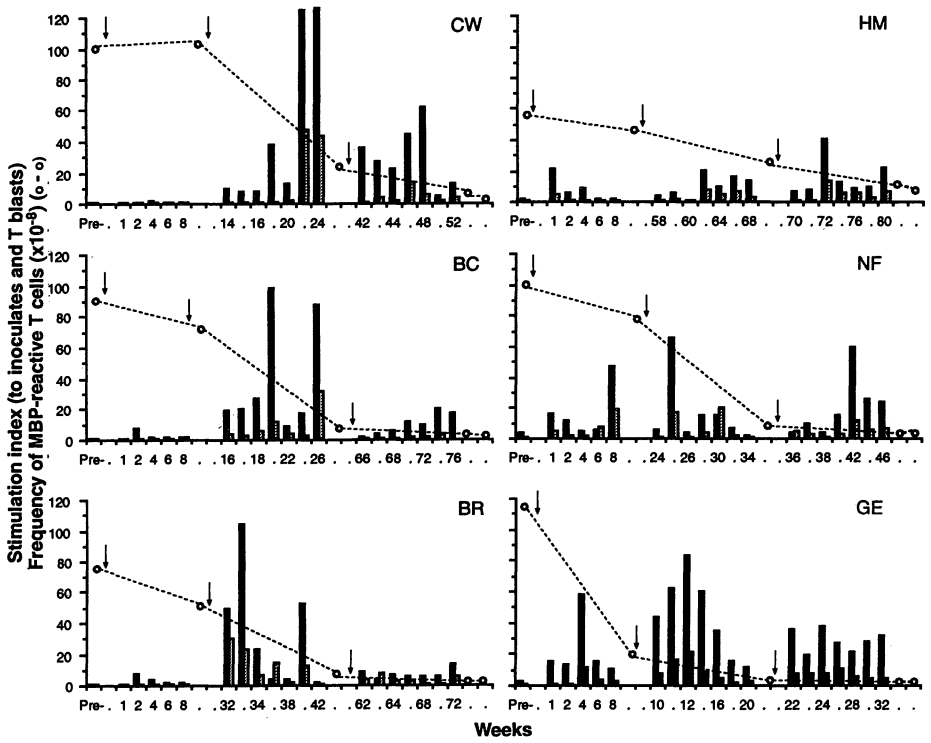

Fig. 1. Proliferative responses to the inoculates and autologous nonspecific T cells generated by PHA and changes in the frequency of MBP-reactive T cells before and after each inoculation. Assays were done before each inoculation and on day 3, week 1, week 2, week 4, week 6, and week 8 after each inoculation as indicated by arrows. Data are given as stimulation indices, defined as the mean counts per minute (cpm) of WMNC plus irradiated inoculates or T cell blasts/the sum of cpm of WMNC cultured alone and cpm of irradiated inoculates or T cell blasts cultured alone (*17*). The frequency of MBP-reactive and TT-reactive T cells was analyzed before and after each inoculation (*17*). Solid bars, inoculate clones; stippled bars, T blasts.

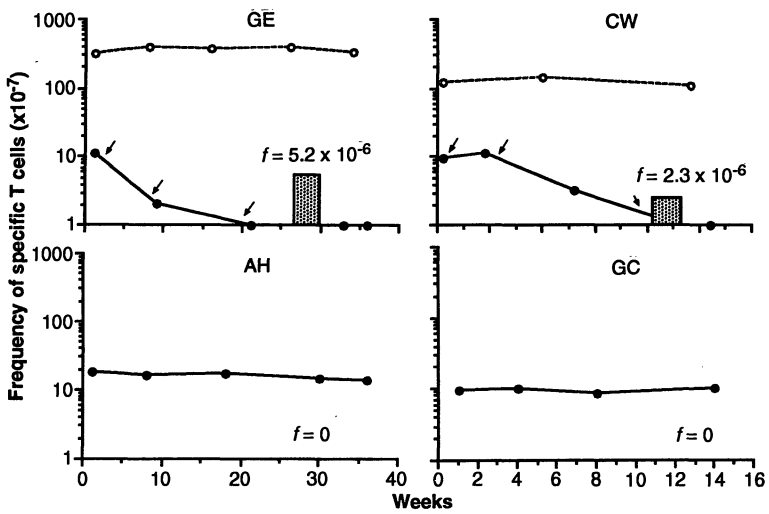

Fig. 2. Relation of changes in the frequencies of T cells reactive to MBP (filled circles), TT (open circles), and the inoculates (stippled bars) in recipient (GE and CW) and in nonrecipient MS subjects (AH and GC) (*17*).

Table 1. Clinical data of the patients and peptide reactivity of MBP-reactive T cell clones used as inoculates (*13*). Values of reactivity to a stimulatory peptide are given in parentheses.

Subject	Age, sex	Diagnosis	Expanded disability status scale (EDSS)	T cell clone	Reactivity (cpm incorporated × 10^3 ± SEM)		
					To MBP	To a stimulatory peptide	To other peptides
BC	43, M	Chronic-progressive	7.0	BC12	6.8 ± 0.5	143–168 (8.2 ± 0.6)	0.2 to 0.4
				BC-6	5.6 ± 0.4	110–129 (3.4 ± 0.1)	0.2 to 0.3
				1B7-E4	42.5 ± 3.1	84–102 (43.8 ± 2.2)	0.1 to 0.6
BR	31, F	Relapsing-remitting	1.0	BR-1	28.7 ± 2.2	143–168 (22.1 ± 2.8)	1.2 to 2.3
				BR-3	16.6 ± 1.8	143–168 (18.4 ± 2.5)	0.8 to 1.1
				1G7	52.3 ± 4.8	143–168 (47.4 ± 5.2)	1.3 to 1.7
HM	47, M	Relapsing-remitting	4.5	1D5	45.8 ± 5.9	143–168 (38.7 ± 4.6)	0.8 to 1.2
				HM-1	78.5 ± 7.9	143–168 (82.2 ± 7.2)	1.4 to 1.8
CW	46, M	Chronic-progressive	7.5	CW-5	17.8 ± 1.6	45–89 (14.4 ± 1.1)	0.1 to 0.4
				CW-10	5.4 ± 0.6	143–168 (7.8 ± 0.8)	0.1 to 0.2
				1E4	37.6 ± 4.8	143–168 (27.3 ± 2.4)	0.1 to 0.3
NF	46, F	Primary-progressive	4.5	C10	32.8 ± 2.6	90–170 (28.2 ± 2.2)	1.1 to 1.4
				1B3	13.1 ± 1.2	90–170 (15.8 ± 1.9)	0.7 to 1.8
GE	26, F	Relapsing-remitting	3.0	GE-2	20.1 ± 1.8	84–102 (11.2 ± 0.8)	1.1 to 1.4
				GE-3	42.2 ± 2.3	84–102 (23.6 ± 2.1)	1.8 to 2.6
				GE-4	37.0 ± 3.2	84–102 (24.8 ± 2.2)	0.8 to 1.9

anti-clonotypic T cells we obtained might represent only part of the T cell populations induced by the inoculation, because the selection was based on their inhibitory effect. Other responder T cells of the CD4 phenotype may act by driving the regulatory network to enhance suppression, as illustrated by so called "anti-ergotypic" T cells isolated from vaccinated experimental animals (*22*).

Monitoring for toxicity over the entire trial confirmed that this inoculation was safe; no side effects were observed and no changes were measured in standard toxicity assays. There was no evidence for acute exacerbations related to the inoculation. However, no conclusion as to treatment efficacy can be drawn from the present study.

The target molecule recognized by these anti-clonotypic T cells has not been defined. Candidate antigens may include the self MHC molecules, T cell activation markers, and the TCR. However, our data would argue against self MHC molecules or T cell activation markers as potential targets, because the responses were directed specifically to the inoculates and not to autologous T cells responding to a recall antigen (TT) and expressing the same MHC molecules and activation markers. Thus, the results obtained suggest that the MHC class I–restricted response is directed to a variable region of the TCR, a feature that serves to distinguish the inoculates from self T cells with irrelevant antigen specificity, though data are not presently available as to where on the TCR such a response is directed.

In support of this possibility, experiments in EAE and adjuvant-induced arthritis indicate that inoculation of autoreactive T cell clones specific for MBP and *Myco-*

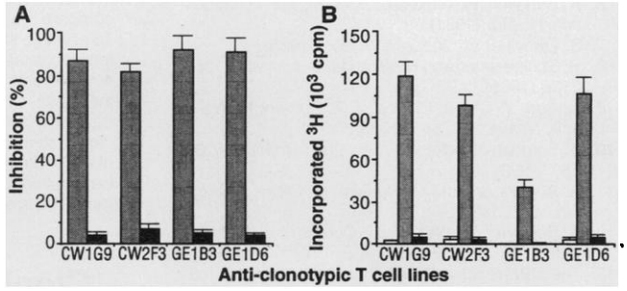

Fig. 3. Specific recognition and inhibition of the inoculates by the anti-clonotypic T cell lines. (**A**) Inhibition assay. Anti-clonotypic T cell lines were irradiated and used as inhibitors. Cells (10^4) were added in triplicate to wells containing 10^4 inoculates or TT-reactive T cells and 10^5 APCs pulsed with MBP or TT in a proliferation assay. Percent inhibition was calculated as in (*18*). (**B**) Stimulation assay. Anti-clonotypic T cell lines were plated at 2 × 10^4 cells per well in triplicate and cultured with 4 × 10^4 autologous inoculates or TT-reactive T cells as stimulators, which were irradiated (8000 rads) to prevent their own proliferation from interfering with the proliferation assays. In all cases, cpm of irradiated stimulators did not exceed 1200. Stippled bars, MBP-specific clones; solid bars, TT-specific clones; open bars, anti-clonotypic T cells alone.

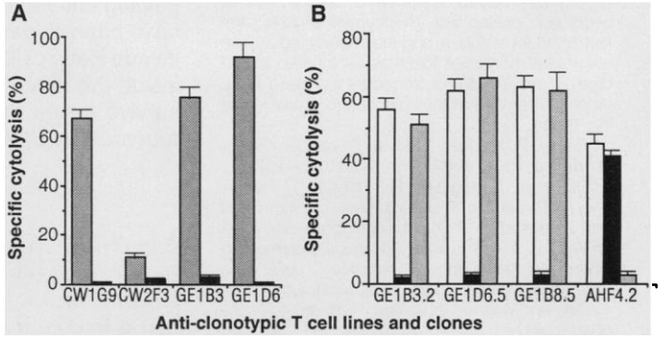

Fig. 4. Specific cytotoxicity and the MHC restriction of the anti-clonotypic T cell lines. (**A**) Cytotoxicity assay. The inoculates or TT-reactive T cells were labeled with 200 μCi of ^{51}Cr for 45 min, washed four times, and used as target cells in a standard chromium release assay. After a 4-hour incubation, supernatants were harvested and measured for radioactivity. The effector (anti-clonotypic T cells) to target (the inoculates and control T cells) ratio was eight. The maximum and spontaneous releases of chromium were determined in wells containing detergent or medium alone. Percent specific cytolysis was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100%. Stippled bars, MBP-specific clones; solid bars, TT-specific clones. (**B**) MHC restriction. Three anti-clonotypic clones were tested for antibody blocking in a chromium release assay. AHF4.2, an MHC class II–restricted CD4$^+$ cytotoxic T cell clone specific for MBP-pulsed target, was used as a control. Effector clones were preincubated with the indicated antibodies at 10 μg/ml for 30 min before mixing with ^{51}Cr-labeled target cells. The effector-to-target ratio was eight. Open bars, medium alone; solid bars, antibody to MHC class I molecules; stippled bars, antibody to MHC class II molecules.

bacterium tuberculosis prevented only the disease that they were able to induce (*23*). Anti-clonotypic responses induced by the inoculates in the same strain of rats were specific for the given T cells and did not cross-react with each other, suggesting recognition of a clonotypic epitope within the TCR. Furthermore, recognition of clonotypic epitopes of the TCR V_β chain of human MBP-reactive T cells leads to specific cytolysis of the target T cells (*10*). The clarification of the target antigen within the TCR will allow future development of "vaccines" that incorporate a peptide matching the target sequences.

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13. The MBP-reactive T cell lines were cloned at 0.3 cell per well by limiting dilution with 10^5 irradiated autologous feeders and PHA (2 μg/ml). Cultures were refreshed with medium containing recombinant interleukin-2 (rIL-2; 5 U/ml) every 3 days. After 12 to 14 days, growing clones were first examined for their reactivity to three fragments of MBP, regions 1 to 37, 45 to 89 and 90 to 170, and subsequently tested with 11 peptides of MBP. Cells (10^4) of each clone were cultured with 10^5 irradiated autologous APCs per well, to which each fragment (10 μg/ml) or each peptide (2 μg/ml) was added. Cells were cultured for 72 hours and pulsed with [^{3}H]thymidine during the last 16 hours of culture and then harvested with an automated harvester to measure tritiated thymidine uptake. The same procedure was used in the other proliferation assays mentioned elsewhere in this report.
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16. The MBP-reactive T cell clones were activated with irradiated MBP-pulsed autologous WMNC at an APC to T cell ratio of 10 and supplemented with rIL-2. Activated MBP-reactive T cells were isolated by Ficoll gradient separation, when necessary, and washed three times with sterile phosphate-buffered saline (PBS) before irradiation (8000 rads, ^{60}Co source). The inoculates were resuspended in 1 ml of PBS and injected subcutaneously (0.5 ml per arm). The number of cells used for vaccination was chosen by an extrapolation of vaccine doses effective in experimental animals on the basis of relative skin surface areas (*24*). All subjects were monitored for changes in counts of granulocytes, lymphocytes, monocytes, and platelets and in prothrombin times, serum glutamic-oxalacetic and glutamic-pyruvic transaminase (SGOP and SGPT), uric acid, blood urea nitrogen, and creatinine at 2-month intervals throughout the inoculation procedure.
17. To assess the proliferative responses, we cultured 5×10^4 fresh WMNC per well in triplicate with 5×10^4 irradiated inoculates or concurrently prepared nonspecific T cell blasts for 72 hours. As a control, WMNC and irradiated inoculates or T cell blasts were cultured alone. Cell proliferation was measured by the proliferation assays (*13*). To estimate the frequency of antigen-reactive T cells, we plated out WMNC at 2×10^5, 10^5, and 0.5×10^5 cells per well for MBP stimulation (MBP, 40 μg/ml) or plated out at 4×10^4, 2×10^4, and 10^4 cells per well for TT stimulation (TT, 2.5 Lf/ml), respectively (60 wells for each concentration). The concentration range was predetermined to allow for sensitive detection (*14*). Cultures were then restimulated with MBP- or TT-pulsed WMNC as a source of APC, and rIL-2 was added at 5 U/ml. After 1 week, each culture was split and tested for specific proliferation to MBP or TT in a proliferation assay. A T cell line was defined as specific when cpm of the wells containing MBP- or TT-pulsed APC/cpm of control wells exceeded 3, and Δcpm was greater than 1000. The frequency of antigen-reactive T cells was estimated by Poisson statistics when the frequency distribution followed the "single-hit" rule (*25*). In cases of "multiple-hit" distribution, the frequency was calculated by dividing the number of specific wells by the total amount of WMNC plated at a concentration that yielded the highest number of specific wells (*14, 15*). For each individual, the same method of calculation was used consistently to compare the frequency changes during the trial.
18. To estimate the frequency of T cells responding to the inoculates, we plated freshly isolated WMNC at 4×10^4 and 2×10^4 cells per well and cultured them with 4×10^4 irradiated inoculates. After 7 days, the cultures were restimulated with the irradiated stimulator T cells and supplemented with rIL-2 (5 U/ml). At day 14, 50% of the cells from each culture were taken out and irradiated at 8000 rads. The irradiated cells were then split into four portions and added in duplicate to culture wells containing 10^4 inoculates or TT-reactive T cells and 10^5 irradiated APCs pulsed with MBP or TT in proliferation assays to measure the inhibitory effect. Percent inhibition was measured as [1 − (proliferation in the presence of irradiated responding T cells as inhibitors/proliferation in the absence of the inhibitor)] × 100%. Cultures exerting more than 60% inhibition on the proliferation of the inoculates were considered as responding cell lines. The frequency was estimated by dividing the number of responding wells by the total WMNC plated (6×10^4 cells). The percentage of responding wells did not exceed 15% in each case.
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Cloning of an *M. tuberculosis* DNA Fragment Associated with Entry and Survival Inside Cells

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Mycobacterium tuberculosis infects one-third of the world's human population. This widespread infection depends on the organism's ability to escape host defenses by gaining entry and surviving inside the macrophage. DNA sequences of _M. tuberculosis_ have been cloned; these confer on a nonpathogenic _Escherichia coli_ strain an ability to invade HeLa cells, augment macrophage phagocytosis, and survive for at least 24 hours inside the human macrophage. This capacity to gain entry into mammalian cells and survive inside the macrophage was localized to two distinct loci on the cloned _M. tuberculosis_ DNA fragment.

Mycobacterium tuberculosis, the etiologic agent of tuberculosis (TB), produces chronic asymptomatic infection in most people that it infects. It sustains chronic infection by entering and surviving indefinitely inside macrophages. In time, and in response to poorly understood cues, the organism proliferates, escapes the macrophages, and causes active TB in a subset of infected persons who become infectious. Resistance to oxidative killing (*1*), inhibition of phagosome-lysosome fusion (*2*), and formation of the so-called electron-transparent zone (ETZ) that impairs diffusion of lysosomal enzymes (*3, 4*) are some of the mechanisms that may explain the survival of *M. tuberculosis* inside macrophages. However, the specific bacterial factors responsible for these properties have not been characterized.

Mycobacterium tuberculosis can invade HeLa cells, which are not phagocytes (Fig. 1) (*5*). Bacterial factors alone are, there-

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