B Cells Turn Off Virgin But Not Memory T Cells

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There are three possible outcomes when a T cell recognizes a cell bearing a self or foreign antigen. (i) The T cell is not sufficiently signaled and is unaffected. (ii) The T cell is activated. (iii) The T cell is turned off. The differentiation state of the T cell is critical to the outcome. Although both virgin and memory T cells can be activated by antigens presented by "professional" antigen-presenting cells such as dendritic cells, they differ in their responses to B cells. Experienced T cells respond to antigen presented by B cells, whereas virgin T cells are rendered tolerant. These findings may relate to the phenomena of low- and high-zone tolerance, neonatal tolerance, and the beneficial effect of blood transfusions on allograft survival.

Although it is clear that "professional" antigen-presenting cells (APCs) such as dendritic cells are potent activators of both virgin (naïve or unprimed) and memory (primed) T cells (*1*, *2*), the activating capacity of B cells has been controversial. Whereas some studies show that B cells (*3*, *4*), especially activated B cells (*5*–*7*), can present antigen to activate T cell hybridomas, T cell clones, and some fresh T cells (*8*), others have reported situations in which B cells were unable to activate T helper cells (*1*, *9*). Sometimes B cells even depress immune responses. For example, spleen cell populations depleted of professional APCs (and therefore enriched in B cells) induce partial tolerance to major histocompatibility complex (MHC) alloantigens in vivo (*10*), and mice are rendered tolerant to rabbit immunoglobulin if the immunoglobulin is injected in a form that is presented specifically by resting (unstimulated) B cells (*11*). Two suggestions have been made to reconcile these apparently contradictory results. (i) The activation state of the B cell may be important in that activated, but not resting, B cells may be able to provide costimulatory signals to T cells (*7*). (ii) The crucial element might instead be a difference in the way in which virgin and memory T cells respond to antigen presented by B cells (*9*). To test these two views, we studied the responses of naïve and memory female T cells to resting and activated B cells expressing the male-specific antigen H-Y. We chose H-Y because there are few if any environmental antigens that cross-react with it (*12*). Thus, the differences between naïve and memory T cells can be accurately distinguished, a situation not found for many other commonly used antigens (*13*).

We first looked at interactions between naïve T cells and resting B cells. C57BL/10 (B10) female mice were injected with resting B10 male B cells that were highly purified, with B10 male spleen cells (containing professional APCs), or with nothing. All the mice were injected with male spleen cells 1 week later, and 2 weeks later they were tested for the generation of cytotoxic T lymphocyte (CTL) responses (Fig. 1). Females first injected with male B cells did not generate CTL responses [nor secrete detectable interleukin-2 (IL-2) or IL-4 (*14*)] to H-Y, whereas mice that had been injected either once or twice with male spleen cells generated efficient CTL responses (Fig. 1, top row). Pooled results from 19 experiments show that 43 of 44 mice given male B cells became unresponsive, whereas 68 out of 80 mice given male spleen cells were immunized (Fig. 1, middle row). The unresponsiveness was H-Y–specific because CTL responses to MHC alloantigens [and to minor H antigens (*14*)] were similar in all of the groups (Fig. 1, bottom row). Thus, resting male B cells, unlike professional APCs, did not elicit CTL responses from naïve T cells. Instead, the outcome was tolerance.

To determine whether B cells could induce transplantation tolerance, we tested the survival of male skin grafts on naïve females, females injected with male resting B cells, and as a positive control, females injected with a preparation enriched for dendritic cells (Fig. 2). All 16 recipients of resting male B cells retained male skin grafts for greater than 100 days, whereas six of six females that received male dendritic cells rejected their grafts quickly. Therefore, the tolerance induced by resting male B cells leads to transplantation tolerance to male tissue grafts.

We next determined how naïve T cells respond to activated B cells, which, unlike

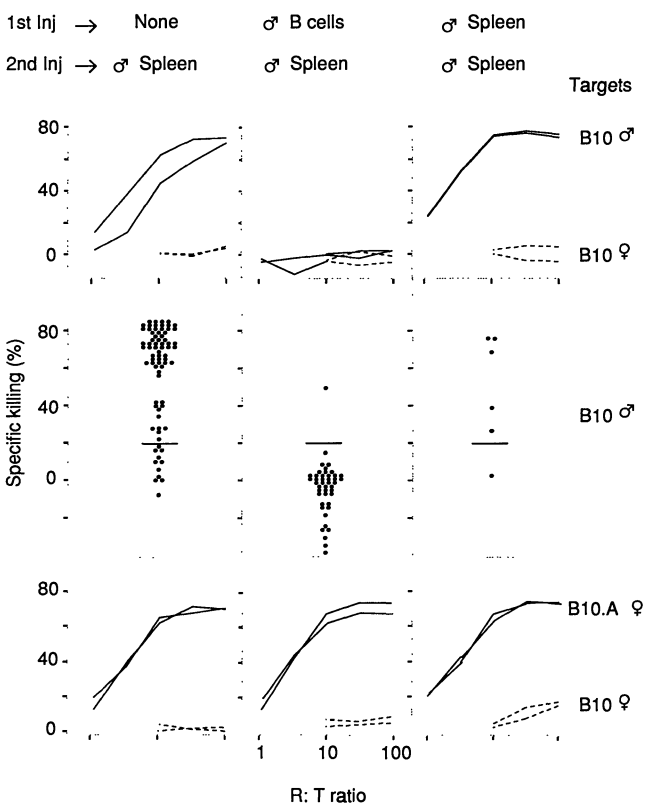

Fig. 1. Intravenous injection of resting male B cells into female recipients induces specific tolerance to the H-Y antigen. The top and bottom rows of panels represent the titrated responses of two individual mice per group tested in a single experiment against target cells from B10 mice bearing the H-Y antigen (top row) and target cells from B10.A mice bearing the H-2^a alloantigens (bottom row); B10.A is an allogeneic strain used as a general control for responsiveness. The middle row represents the responses against H-Y–bearing cells of 124 mice tested in 27 experiments. B10 females (6 to 12 weeks old) received no injection, 10^7 purified B10 male B cells (*31*), or 10^7 unseparated B10 male spleen cells intravenously (IV), as indicated above each column. All mice were primed with an intraperitoneal (IP) injection of 10^7 male spleen cells 1 week later. Recipient spleen cells were stimulated in vitro with B10 male or B10.A spleen cells 2 weeks later and tested 5 days later for killing on B10 male, B10 female, or B10.A targets. Each point in the middle row represents the specific lysis generated by spleen cells from an individual mouse at a responder-to-target ratio (R:T) varying from 30:1 to 133:1. These R:T ratios, determined on the basis of the number of cells originally cultured, were chosen in each experiment to correspond to the point where the responses of the control mice drop off the plateau of specific lysis.

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resting B cells, can be effective stimulators of primary allogeneic responses in vitro (6). To circumvent the effects of activated professional APCs, we purified the B cells before stimulating them with lipopolysaccharide (LPS). We found that these LPS-stimulated blasts were nearly as potent as spleen cells in their ability to stimulate Rachel, an H-Y–specific T helper clone and that this ability was resistant to high doses of irradiation, a characteristic of activated but not resting B cells (Fig. 3) (*15*). Nevertheless, the LPS-stimulated blasts induced tolerance to H-Y when injected into naïve female mice. By titration, activated B cells were three times as efficient as resting B cells in their capacity to induce tolerance in vivo (*14*). Therefore, naïve T cells did not discriminate between resting and activated B cells. When professional APCs were removed, both resting and activated B cells induced unresponsiveness to H-Y.

Finally, we evaluated the effect of B cells on memory T cells. We immunized a group of naïve female mice with male spleen cells and tested each mouse for its reactivity to H-Y. We then injected these primed mice and a group of unprimed mice with 10^7 or 10^8 purified male B cells and retested them later for responsiveness to H-Y. The responses of the primed animals were undiminished by even large doses of B cells (Fig. 4, groups 3 and 4); in fact, most of the responses (expressed as lytic units per million spleen cells) increased from 2- to more than 35-fold. Thus, the same population of B cells induced tolerance in naïve T cells (group 2) and activated memory T cells.

These data demonstrate two rules governing the outcome of T cell–B cell interactions. (i) A T cell's reaction after encounter with a B cell depends on that T cell's history; naïve cells are turned off, whereas memory cells are reactivated. (ii) Both resting and activated B cells are tolerogenic for naïve T cells.

Although there are no a priori reasons why B cells should be tolerogenic APCs for naïve T cells, there is at least one reason why they should not be stimulatory (*9*). The immune system, in trying to respond to potential foreign antigens while maintain-

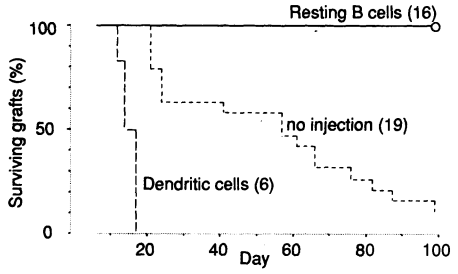

Fig. 2. Induction of transplantation tolerance by injection of resting B cells. B10 females received no injection, 10^7 resting male B cells injected IV, or 3 × 10^5 purified male dendritic cells injected IV (*31*). All mice were grafted on the left flank with a piece of tail skin (5 mm by 5 mm) 1 week later. Bandages were removed 7 days later, and grafts inspected thrice weekly. Rejection was scored when no viable grafted tissue remained. Numbers of mice per group are indicated in parentheses.

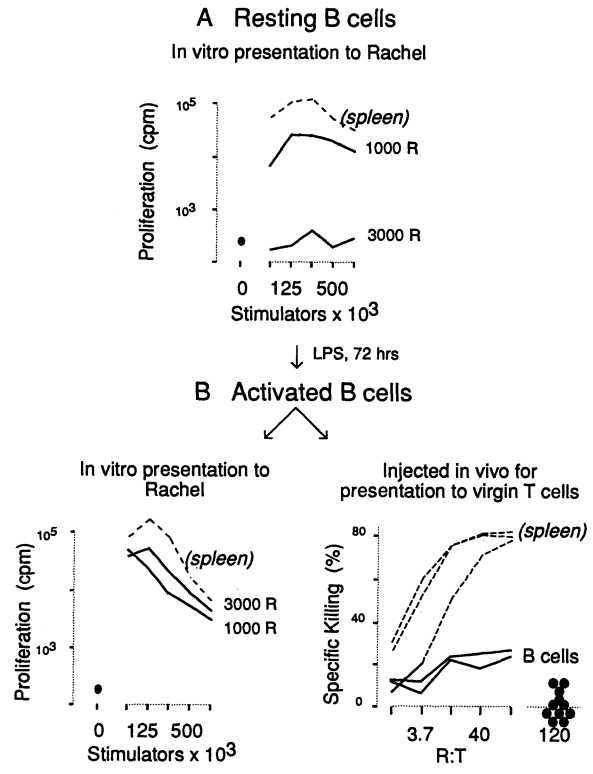

Fig. 3. Activated B cells induce tolerance to the male antigen. Resting and activated B cells were tested for their ability to stimulate Rachel, a CD4+ T cell clone, and for their effect when injected in vivo. (**A**) In vitro APC function of resting B cells is radiosensitive. Rachel cells were stimulated with graded numbers of high-density resting B cells (*31, 32*) that had been irradiated with 1000 or 3000 R or, as controls, with T cell–depleted male spleen cells that had been irradiated with 3000 R. Cross-hatched circles indicate the proliferation of Rachel in the absence of stimulator cells. (**B**) Activation does not affect the capacity of B cells to induce tolerance to H-Y in vivo. B10 female mice (two to four per group) were left uninjected or were injected IV with 10^7 high-density B cells (96.7% B220+) or with 10^7 LPS-activated B cells (98.3% B220+). One week later, all animals received 2 × 10^6 B10 male spleen cells IP, and 2 to 4 weeks later they were tested for killing activity to H-Y. Shown is the killing against B10 male targets. Filled circles (bottom right) are a summary of the responses of ten mice from three experiments injected with 10^7 B10 male LPS-stimulated blasts 1 week before priming with 10^7 B10 male spleen cells. Killing of syngeneic female targets at the same R:T ratio was subtracted. All animals gave good and equivalent killing of B10.A targets (*14*).

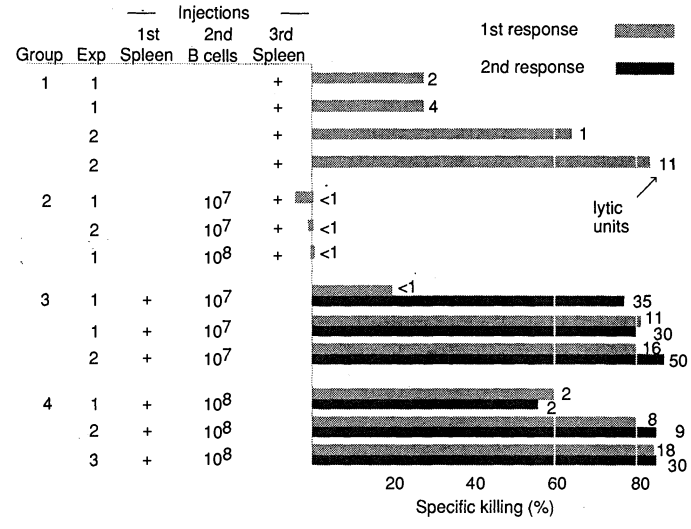

Fig. 4. Female mice immunized to H-Y are not rendered tolerant by a subsequent injection of male B cells. B6 or B10 female naïve mice were left uninjected (group 1) or injected IV with 10^7 or 10^8 B10 male resting B cells (group 2). Later (1 to 2 weeks), all animals in groups 1 and 2 received 2 × 10^6 to 10 × 10^6 male whole spleen cells IP. After 2 weeks, they were tested for reactivity to H-Y (*32*). For groups 3 and 4, female mice were primed with 2 × 10^6 to 10 × 10^6 whole male spleen cells IP and rested for 2 (experiments 2 and 3) or 16 (experiment 1) weeks. They were then hemisplenectomized (under ketamine-xylazine anesthesia), and their spleen cells were tested for reactivity to H-Y (light gray bars). After 1 to 2 weeks, they were injected with 10^7 (group 3) or 10^8 (group 4) of the same B cells used in group 2. Cells from the remaining spleen fragment were tested for the generation of killers against H-Y (dark gray bars) 1 to 4 weeks later. Bars represent maximum killing by individual animals, and the numbers to the right of bars are lytic units. Lytic units equal 1 × 10^6 divided by the number of input spleen cells required to generate half the maximum specific lysis obtained in the experiment.

ing self tolerance, has a problem with B cell idiotypes. Tolerance to every idiotype would cripple the system, but the ability to respond could result in polyclonal activation of both B and T cells. Jerne's solution to this problem led him to the network hypothesis (*16*). He proposed that the circulating amount of any particular idiotype was too low to immunize or induce tolerance and that it increased to immunizing concentrations only when a B cell responded to a foreign antigen. What Jerne did not take into account, however, is that the concentration of any particular idiotype, though low in circulation and therefore infrequently presented by professional APCs, is high enough on the B cell that makes that particular antibody for the idiotype to be presented to T cells (*17*). If B cells were able to deliver costimulatory signals, there would be antiidiotype responses even in unimmunized animals. Such a potentially dangerous situation is avoidable if neither resting nor activated B cells (because both express idiotypes) are able to deliver the appropriate costimulatory signals necessary to activate naïve T cells. If, as evidence suggests, T cells are turned off by recognition of antigen in the absence of costimulation (*18*), tolerance would be the unintended consequence of a B cell's inability to deliver costimulatory signals to naive T cells.

What about the evidence that shows that B cells can activate T cells? We had suggested (*9*) that B cells should be able to restimulate memory T cells in order to efficiently maintain ongoing immune responses as the amount of antigen dwindled, and with three exceptions, the evidence is that B cells are stimulatory in vivo and in vitro for primed T cells (*3–7*) but not for naïve T cells (*9–11*). The first exception is the case of T cells responding to MHC or Mls antigens, where B cells have been reported to activate (*6*), not activate (*1*), or induce tolerance (*10, 19*). Because these T cells are likely to be mixtures of naïve cells and memory cells that have been cross-primed by environmental antigens (*13*), presentation of MHC or Mls antigens by B cells should initiate two opposing reactions: tolerance of the naïve T cells and reactivation of the cross-primed memory cells. The end result for any antigen would thus depend on the proportions of naïve and memory T cells, as well as on the number of contaminating professional APCs remaining in the B cell preparation.

The second exception is that activated B cells can act as costimulators for activation of naïve T cells mediated by anti-CD3 (*7*). In this in vitro study, the authors used the CD45R cell surface marker to separate $CD4^+$ T cells into naïve ($CD45R^+$) and memory ($CD45R^-$) populations. However, because $CD45R^-$ cells can convert to the $CD45R^+$ phenotype (*20*), CD45R may not reliably distinguish between naïve and memory cells.

The third exception is that naïve mice injected with monovalent Fab fragments of rabbit antibody to mouse immunoglobulin D (IgD) became unresponsive to rabbit Ig, whereas mice injected with divalent $F(ab')_2$ fragments of the same antibody did not (*11*). Because $F(ab')_2$ fragments can activate B cells by cross-linking surface Ig, the authors proposed that resting, but not activated, B cells were tolerogenic. Because IgD circulates at low concentrations, however, an alternative possibility is that the anti–IgD-$F(ab')_2$ fragments were actually presented by professional APCs that had captured IgD–anti-IgD complexes, complexes that could not be made by Fab fragments (*21*).

The finding that B cells induce tolerance in naïve T cells but immunize memory T cells offers an explanation for high- and low-zone tolerance (*22*). Because B cells, through their antigen-specific surface Igs, are thousands of times more efficient at capturing specific antigen than are other APCs (*4*), antigens at very low concentrations would be presented mainly by antigen-specific B cells, which would induce tolerance in responding T cells. At medium concentrations, the antigen would be presented by professional APCs, which outnumber antigen-specific B cells, and should compete effectively with the B cells to immunize the T cells. At very high concentrations, the antigen would be captured nonspecifically by all B cells, and because B cells far outnumber professional APCs, the result would again be tolerance. By the same reasoning, B cell presentation of transplantation antigens may also account for the initial induction of tolerance in neonatal mice (*23*) because neonatal T cells exist in low numbers and are likely to be in a virgin state.

Tolerance induction by superantigens can also be explained by the tolerogenic effect of B cells on T cells. Injections of high doses of bacterial superantigens or of cells bearing endogenous viral superantigens lead to tolerance and a reduction of the numbers of specific T cells rather than to an increase in frequency. Many of the endogenous superantigens are expressed by B cells but not by professional APCs (*24*), and the exogenous superantigens, because they bind directly to MHC class II molecules, would be presented by the majority of B cells, creating a situation analagous to high-zone tolerance, where the B cells, effectively outnumbering professional APCs, induce tolerance in the virgin T cell population (*25*).

Induction of tolerance by B cell can also explain the beneficial effect of donor-specific blood transfusions. Blood transfusions in humans (*26*) and intravenous injections of APC-depleted spleen cells in rodents (*10*) elicit prolonged graft acceptance though rarely a state of complete tolerance. The dearth of professional APCs and the large number of B cells in blood may induce tolerance in the naïve alloreactive T cells of the recipient and thus significantly reduce the response. As outlined above, however, any memory cells in the responding populations would not become tolerant, and any contaminating professional APCs would immunize rather than induce tolerance (*2*). There is also the matter of tissue-specific antigens. If the allogeneic response is actually specific for a plethora of MHC-peptide complexes (*27*), then B cells would only induce tolerance in those T cells specific for the MHC complexes they express, leaving T cells that recognize kidney-specific or skin-specific antigens to generate the remaining activity seen in these cases.

Because naïve and memory cells respond differently to B cells but professional APCs can stimulate both, there must be a difference in the costimulatory signals sent by the presenting cells and in the corresponding receptors on the responding T cells. The study of activation requirements for virgin T cells may therefore reveal distinct cellular pathways from those that have been demonstrated in T cell clones and hybridomas.

Finally, our data provide evidence for a distinct second stage of T cell tolerance susceptibility. The first stage occurs in the developing thymocyte, which may be rendered tolerant by being exposed to antigen on many different cell types, including professional APCs (*28*). The second stage occurs in the naïve T cell, which can become tolerant either by having its antigen presented to it in the absence of costimulatory signals from a professional APC or by having its antigen presented to it in the absence of T cell help (*29*). Is there a third stage in which memory cells can become tolerant? These are the cells that remain the major challenge to transplantation immunologists, and there is some indication that they too may be susceptible to tolerance induction (*18, 30*).

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21. A distinction between the two explanations could be made with a chimeric animal carrying two populations of B cells and APCs, one bearing MHC A and IgD allotype A and the second bearing MHC B and IgD allotype B. An injection of anti-IgD A should prime T cells restricted by MHC type A if the priming is done by the targeted B cells and should prime T cells restricted by both MHC types if the priming is a result of the presentation of captured IgD–anti-IgD complexes by professional APCs.
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31. Cell preparations were done in serum-free medium. We have found that a tiny number of contaminating dendritic cells is sufficient to immunize rather than induce tolerance in a certain proportion of female mice. Thus, unless care is taken to rigorously remove these cells, many populations considered to be B cells will immunize because of contaminating professional APCs. To purify B cells, we depleted spleens from B10 male mice of red cells with ammonium chloride buffer and of T cells with antibodies to Thy1.2 (J1j.10), CD4 (RL172.4), and CD8 (3.155) and guinea pig complement. After passage over two sequential Sephadex G10 columns (Pharmacia), the cells were centrifuged over discontinuous Percoll gradients containing 60% and 70% layers. Cells at the 60 to 70% interface were used for injection. B cells prepared this way were 96.7 to 99.7% $B220^+$, <1% $CD4^+$ or $CD8^+$, <1% $NK1.1^+$, and were considered free of macrophages or dendritic cells by virtue of their inability, when given 3000 R irradiation, to stimulate allogeneic mixed-lymphocyte reactions or the proliferation of T cell clones (Fig. 3) (*14*). For LPS-stimulated blasts, purified resting B cells were cultured (4×10^6 cells per 2-ml well) for 72 hours in complete medium (CM) containing LPS (50 mg/ml) (*Escherichia coli* O55:B5, Difco, Detroit, MI) and washed four times in phosphate-buffered saline (PBS) before further use. To purify dendritic cells, we layered spleen suspensions onto plastic dishes (2 spleens per dish; Falcon, Lincoln Park, NJ), in 7 ml of Eagle's Hanks amino acid medium (EHAA) and 1% normal mouse serum (NMS). After 90 min at 37°C, nonadherent cells were removed, the plates were rinsed, and the medium was replaced. After another 8 hours at 37°C, nonadherent cells were collected and centrifuged over a 50% Percoll gradient. Buoyant cells were recovered, washed in EHAA and 1% NMS, and resuspended in PBS for injection. By fluorescence analysis, cells were 83% $33D1^+$ (a dendritic cell-specific marker).
32. For proliferation, 10,000 Rachel cells were stimulated in 0.2-ml cultures with graded numbers of stimulators for 48 hours. ^{3}H-thymidine (5 μCi/ml) was added for the last 12 hours. For killing, 4×10^6 responder spleen cells were cultured for 5 days against 2×10^6 irradiated (3000 R) stimulator spleen cells in 2 ml of EHAA medium containing 10% fetal calf serum, 50 mM 2-mercaptoethanol, and antibiotics (CM). They were then harvested and tested for cytotoxic activity by the JAM Test, an assay for CTL activity [P. Matzinger, *J. Immunol. Methods* **145**, 185 (1991)].

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TECHNICAL COMMENTS

Perceptual Correlates of Massive Cortical Reorganization

T. P. Pons *et al.* (*1*) found cortical maps to be capable of an unexpectedly large degree of reorganization. After long-term (12 years) deafferentation of one limb in adult primates, the cortical area of the brain corresponding to the limb became responsive to stimuli applied to the lower face region. This finding extended the previously recognized maximum area of cortical reorganization in adult primates from a mediolateral distance of 1 to 2 mm to about 10 to 14 mm. Because cells that originally received information from the arm can later receive input from the face, we wondered whether stimuli applied to the face would be mislocalized to the arm. To explore this, we have studied (*2*, *3*) localization of touch sensations in two human patients after amputation of one upper limb and in one patient after amputation of one digit.

We applied light touch (using a cotton swab) or deep pressure to different points on the normal body surface. Stimulation of points even remote from the amputation line evoked precisely localized referred sensations in the phantom limb. We could plot "reference fields," small regions of skin surface that evoked referred sensations in specific parts of the phantom limb (for example, the digits). Our main experimental findings may be summarized as follows:

(i) Points (reference fields) were not randomly distributed. There were two clusters, one on the same side of the face as the phantom limb and one around the line of amputation. Furthermore, there was a precise one-to-one correspondence between these points and those on the phantom limb (*3*). (ii) Sensations were referred most often to the hand, especially to the digits with an overrepresentation of the thumb and "pinky." This may reflect the high cortical magnification of these areas. (iii) The referred sensations were modality-specific; for example, a drop of warm water trickling down the face was felt as "warm water trickling down" in the phantom hand. (iv) Reference fields were somatotopically organized. We suggest that this is a direct consequence of the remapping observed by physiologists (*1*). (v) There was a vivid persistence or short-term "memory" of complex sensations; when we gripped and released the finger adjacent to an amputated finger the patient felt the phantom finger being "gripped,"