experiments did not develop tumors (Table 1). Thus, we have demonstrated that the in vivo activation of T cells by anti-CD3 can result in the enhancement of an antitumor response, the prevention of malignant tumor growth, and lasting tumor-associated immunity. In fact, it is now clear that the immunity is tumor-specific (*4*).

Although we did not observe immunosuppression after administration of low doses of anti-CD3, the potential for inadvertent immunosuppression is always present. The 40-μg dose of anti-CD3 caused enhanced tumor outgrowth (Fig. 3) with earlier animal death. This enhanced growth may be due to the immunosuppression that correlates with the reduction of T cell surface CD3 expression following treatment with 40 μg of anti-CD3. The use of several different activating MAbs that are not immunosuppressive when administered in the appropriate regimen may ultimately be the best approach to optimal immunoenhancement without immunosuppression.

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T Cell CD3-ζη Heterodimer Expression and Coupling to Phosphoinositide Hydrolysis

MLADEN MERĆEP, JUAN S. BONIFACINO, PILAR GARCIA-MORALES, LAWRENCE E. SAMELSON, RICHARD D. KLAUSNER, JONATHAN D. ASHWELL

The T cell antigen receptor consists of an antigen-binding heterodimer that is noncovalently associated with at least five CD3 subunits (γ, δ, ε, ζ, and η). The CD3-ζ chains are either disulfide-linked homodimers (CD3-ζ_2) or disulfide-linked heterodimers with η (CD3-ζη). Variants of a murine antigen-specific T cell hybridoma that express normal amounts of CD3-ζ_2 but decreased amounts of CD3-ζη were isolated. When activated, the parental cell line increased both phosphatidylinositol hydrolysis and serine-specific protein kinase activity to a much greater extent than the variants. In contrast, the activation of a tyrosine-specific kinase after stimulation with a cross-linking antibody to CD3 was similar among these cells. There was a positive linear relation between the expression of CD3-ζη and phosphoinositide hydrolysis stimulated by the TCR, suggesting a differential coupling of the T cell αβ heterodimer to signal transduction mechanisms due to αβ association with either CD3-ζ_2 or CD3-ζη.

THE T CELL ANTIGEN RECEPTOR (TCR) consists of an antigen-binding heterodimer noncovalently associated with at least five nonpolymorphic subunits of CD3: γ, δ, ε, ζ, and η (*1, 2*). The large majority (~90%) of the CD3-ζ chains exists as disulfide-linked homodimers, CD3-ζ_2, with the remainder being disulfide-linked heterodimers with η, CD3-ζη (*2*). The gene encoding the CD3-ζ chain has been cloned (*3*); its product is predicted to have a single cysteine residue available for covalent interactions. Therefore, a CD3-ζ chain is either disulfide-linked to a second CD3-ζ chain or a CD3-η chain, but not both. This has suggested a model in which two structurally distinct TCR complexes exist, the predominant one containing CD3-ζ_2 and the remainder containing CD3-ζη (*2*).

T cells bearing variant TCR complexes were produced by the subcutaneous injection of syngeneic mice with 2B4.11 T hybridoma cells and then intraperitoneal injections of monoclonal antibody (MAb) 2C11 (anti–CD3-ε) (*4*). Activation of 2B4.11 T cells in vitro with this MAb leads to a cell cycle block and autolysis (*5*). Despite treatment with anti–CD3-ε in vivo, tumors appeared at the site of inoculation in two different mice. The tumors were excised, and the cells were cultured and subcloned by limiting dilution (*6*). Since 2B4.11 cells frequently lose expression of CD3-ζ (*7, 8*), the subclones were analyzed by immunoblotting with an antiserum to CD3-ζ (anti–CD3-ζ). All five subclones obtained from one tumor line expressed little or no CD3-ζ chain and had low amounts of cell surface TCR, consistent with a previous analysis of a CD3-ζ–deficient 2B4.11 variant (*7*). The second tumor line was heterogeneous in its expression of cell surface TCR, and many subclones from this line expressed normal amounts of CD3-ζ. Two cells, EV.3 and 2M.44, from separate subclonings of this tumor were further analyzed.

Both anti–CD3-ε and a MAb to the 2B4 TCR-α chain, A2B4-2 (*9*), were used to evaluate cell surface expression of TCR (Fig. 1). The 2B4.11 cell line and both variants had single peaks of fluorescence, with 2B4.11 > 2M.44 > EV.3 (EV.3 cells had ~60 to 75% the mean fluorescence intensity of 2B4.11 cells on multiple analyses). Solu-

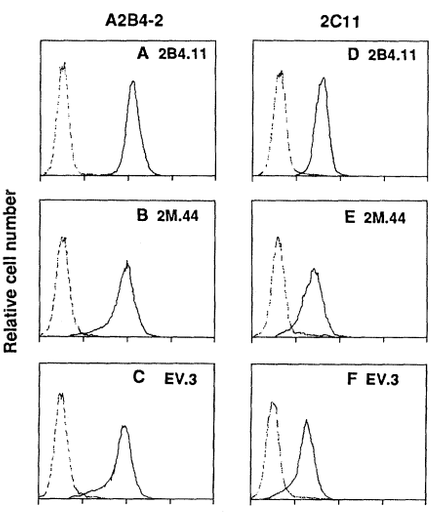

Fig. 1. Cell surface expression of TCR by 2B4.11 and two variants. 2B4.11, 2M.44, and EV.3 were analyzed by indirect immunofluorescence flow cytometry for cell surface expression of the TCR-α chain (**A** to **C**) or the CD3-ε chain (**D** to **F**) (solid lines). Background immunofluorescence was determined by staining with the fluoresceinated F(ab')$_2$ second antibody alone, either goat antibody to mouse immunoglobulin or goat antibody to hamster immunoglobulin, respectively (dotted lines). The 2B4.11 and EV.3 cells stained weakly or not at all with anti-L3T4, and both cells stained brightly with antibodies to Thy-1 and Ly-6 (*10*).

M. Merćep and J. D. Ashwell, Biological Response Modifiers Program, National Cancer Institute, Bethesda, MD 20892.
J. S. Bonifacino, P. Garcia-Morales, L. E. Samelson, R. D. Klausner, Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, Bethesda, MD 20892.

bilized cell membranes were labeled with ^{125}I, the TCR complexes were immunoprecipitated with anti–CD3-ε or anti–CD3-ζ, and resolved by two-dimensional SDS–polyacrylamide gel electrophoresis (PAGE) with the second dimension run in the presence of reducing agent (Fig. 2A). 2B4.11 and EV.3 expressed equivalent amounts of coprecipitated α, β, γ, δ, ε, and ζ. CD3-η was clearly precipitated from 2B4.11 cells, and is visible as a band (22 kD) directly above its disulfide-linked partner, CD3-ζ (16 kD) (Fig. 2A, left panels). In contrast, despite equivalent amounts of directly precipitated CD3-ζ, the CD3-η chain was barely detectable in EV.3 cells (Fig. 2A, right panels). Similar results were obtained with 2M.44 cells (*10*). The amount of CD3-ζη in these cells was quantified by immunoblotting of twofold serial dilutions of immunopurified TCR with anti–CD3-ζ. Under nonreducing conditions, CD3-ζ_2 migrates at 32 kD, whereas CD3-ζη migrates at 38 kD. Thus, while all the cells expressed similar amounts of CD3-ζ_2, the levels of CD3-ζη were markedly reduced in EV.3 and 2M.44 cells compared to 2B4.11 (Fig. 2B). By quantitative densitometry, the fractions of total cellular CD3-ζ covalently linked to CD3-η in 2B4.11, EV.3, and 2M.44 cells were 11, 0.8, and 1.7%, respectively.

The possible functional consequences of a deficiency in CD3-ζη expression were studied by analyzing responses to TCR-dependent stimuli. An early event in activated T cells is the hydrolysis of phosphoinositides to water-soluble inositol phosphates (IP) (*11*), a phenomenon that is well documented in 2B4.11 cells (*12, 13*). Antigen- or anti–CD3-ε–stimulated 2B4.11 cells generated large amounts of IP in response to either stimulus, whereas both CD3-ζη–deficient cells responded poorly (Fig. 3, A and B). The maximal amount of IP generated by 2M.44 was 10% (2C11) and 5% (antigen), and for EV.3 was 3% (2C11) and 1% (antigen), of that generated by 2B4.11 cells. The cells were also incubated with AlF_4^-, a direct activator of guanosine triphosphate (GTP)–binding proteins and subsequent phosphoinositide hydrolysis in a number of cell types, including T cells (*12, 14*). Although AlF_4^- generated substantially less hydrolysis of phosphoinositide than did either antigen or antibodies to the TCR, it resulted in easily measurable IP production by all three cells (Fig. 3C). Notably, the response of EV.3 was similar to that of 2B4.11, while 2M.44 produced even more IP than 2B4.11 cells. Therefore, despite their poor response to antigen or anti–CD3-ε, the CD3-ζη–deficient cells responded as well as the wild-type cell to a stimulus that is thought to bypass the surface TCR.

One consequence of phosphoinositide hydrolysis is the generation of diacylglycerol

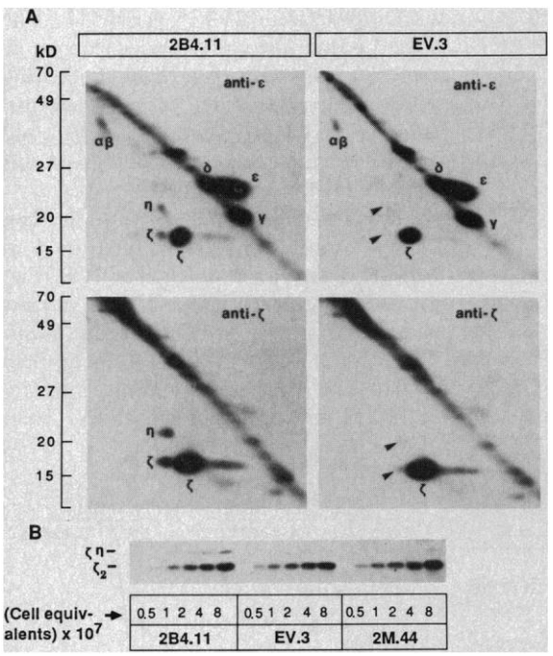

Fig. 2. Expression of CD3-ζη by 2B4.11, EV.3, and 2M.44 cells. (**A**) Immunoprecipitation of ^{125}I-labeled TCR chains. Total membranes from 2B4.11 and EV.3 cells were prepared and solubilized (*18*), and lysates were labeled with ^{125}I by the lactoperoxidase-glucose oxidase method. TCR complexes were isolated by immunoprecipitation with anti–CD3-ε (MAb 2C11) or anti-ζ (antiserum 124), as indicated, and resolved by two-dimensional nonreducing (horizontal)/reducing (vertical) SDS-PAGE. The positions of the TCR chains are indicated. Arrowheads, the CD3-ζη heterodimer. (**B**) Immunoblot of the CD3-ζ chain. Cells were extracted with 0.5% (w/v) Triton X-100, 0.3*M* NaCl, 50 m*M* tris-HCl, *p*H 7.4, and the detergent-solubilized proteins incubated for 1 hour at 4°C with anti–CD3-δ–Sepharose to isolate TCR complexes. Immunoprecipitates were resolved by SDS-PAGE under nonreducing conditions and transferred to nitrocellulose (*19*). The ζ chains were detected by immunoblotting with anti–CD3-ζ and ^{125}I-labeled protein A (*2*). The positions of the ζη heterodimer and the ζ_2 homodimer are indicated.

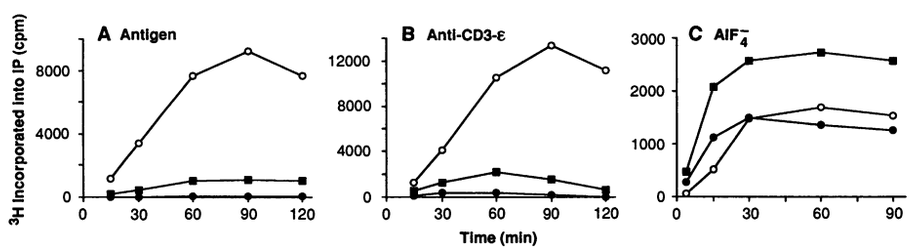

Fig. 3. Generation of water-soluble inositol phosphates upon activation. Cell lines 2B4.11 (○), EV.3 (●), or 2M.44 (■) were loaded with myo-[^3H]inositol and stimulated with (**A**) 10 μ*M* pigeon cytochrome c fragment 81–104, (**B**) 3.3% anti–CD3-ε culture supernatant, or (**C**) AlF_4^- (75 m*M* NaF and 10 μ*M* $AlCl_3$). At the indicated times, the cells were lysed, the lipid was extracted, and the water-soluble labeled products of phosphoinositide hydrolysis were measured by anion exchange chromatography (*7*). For measurement of TCR-mediated activation, 10^6 labeled T cells per experimental point were added in duplicate to tubes containing 10^6 LK 35.2 cells (*20*) with or without pigeon cytochrome c fragment 81–104 (10 μ*M* final concentration) or LK 35.2 cells (10^6) with or without anti–CD3-ε supernatant (3.3% final concentration). IP generation in response to AlF_4^- was measured by incubating 3×10^6 cells with $AlCl_3$ (10 μ*M*) and NaF (75 m*M*), or medium alone. The amount of ^3H incorporated into lipid by each cell was comparable (within 10%) within each experiment. To calculate specific radioactivity, the number of counts per minute (cpm) achieved at each time point in medium alone was subtracted from that obtained with the different stimuli.

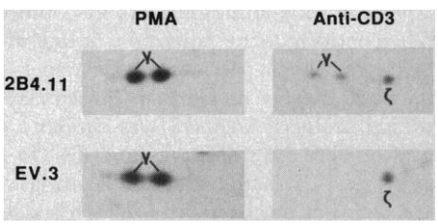

Fig. 4. Anti–CD3-ε–induced phosphorylation as a function of CD3-ζη expression; two-dimensional electrophoresis of phosphorylated CD3-subunits. In two separate experiments 2B4.11 and EV.3 cells were labeled with [^{32}P]orthophosphate (*15*). Radiolabeled cells (5×10^7) were activated with either PMA or LK 35.2 cells (*20*) plus a 4% final concentration of anti–CD3-ε supernatant. Cells were washed, solubilized in nonionic detergents with protease and phosphatase inhibitors (*15*), the nuclei were removed, and supernatants immunoprecipitated with the A2B4-2 MAb adsorbed to protein A agarose. The immunoprecipitated TCR was subjected to two-dimensional nonequilibrium *p*H gel electrophoresis (NEPHGE)/SDS-PAGE and autoradiography. The origin of the tube gel (acidic end) is at the right side of each gel. Phosphorylated receptor subunits are as indicated.

(DAG), an endogenous activator of protein kinase C (PKC), which participates in the activation-induced phosphorylation of the CD3-γ chain (*15*). Activation with anti–CD3-ε resulted in an increase in ^{32}P-labeling of CD3-γ in 2B4.11 cells. In contrast, EV.3 cells had little enhancement of the ^{32}P-labeling of CD3-γ. This could not be explained by a primary defect in either PKC or substrate, since exposure of both cell lines to a chemical activator of PKC, phorbol myristate acetate (PMA), caused equivalent phosphorylation of CD3-γ (Fig. 4).

In addition to the PKC-dependent phosphorylation of CD3-γ, activation of 2B4.11 cells with anti–CD3-ε activates a tyrosine-specific kinase to phosphorylate CD3-ζ (*16*). Activation of EV.3 cells resulted in phosphorylation of CD3-ζ that was quantitatively similar to the parental 2B4.11 cells (Fig. 4). When normalized for cell surface TCR expression, EV.3 cells displayed a dichotomy between TCR-mediated CD3-ζ phosphorylation (96% of 2B4.11 cells) and phosphoinositide hydrolysis (7% of 2B4.11 cells) (*17*). Together, these data indicate that MAb-mediated stimulation via ζη-deficient receptor complexes resulted in relatively normal tyrosine kinase activation, but little enhancement of PKC activity.

An independently derived variant of 2B4.11, Tαβ1.2, is impaired in phosphoinositide hydrolysis and generation of increased intracellular Ca^{2+} upon activation (*13*). We evaluated the presence of CD3-ζη in these cells. By quantitative immunoblotting with anti–CD3-ζ, we found that CD3-ζη expression in Tαβ1.2 T cells was 60% lower than in 2B4.11. Three other 2B4.11 subclones (2M.17, 2M.51, and 2M.143) that were derived at the same time as 2M.44, and whose cell surface TCR amounts were similar to those of 2B4.11 cells, were subjected to quantitative immunoblotting and found to express levels of CD3-ζη that ranged from 71 to 115% of 2B4.11. The maximal amount of IP produced by each cell was plotted as a function of the percentage of CD3-ζη it expressed (with the levels expressed by 2B4.11 being defined as 100%); the analysis revealed a linear relation with the slopes of the linear regression lines close to 1, suggesting that CD3-ζη expression was a limiting factor in determining the amount of phosphoinositide hydrolysis generated after stimulation via the TCR (Fig. 5).

Our results permit us to tentatively assign relations between the structure of the TCR and the proximal biochemical pathways to which it is coupled. One manifestation of TCR-stimulated gene expression is the production of lymphokines. Unlike 2B4.11 cells, both EV.3 and 2M.44 cells produced only low levels of interleukin-2 (IL-2) when stimulated with cross-linked anti-TCR (generally <5% of that produced by 2B4.11). This result is difficult to evaluate, however, because these cells also produced comparably little IL-2 in response to PMA and ionomycin, chemical stimuli that are thought to bypass the TCR. The activation-induced inhibition of spontaneous growth, a response distinct from IL-2 production (*5, 8*), was also assessed. All cells responded to TCR-mediated stimulation with decreases in [^{3}H]thymidine incorporation (*10*), demonstrating that the CD3-ζη–deficient receptors were capable of transducing the signals necessary for at least some cellular responses.

The simplest model that may explain our results is that the TCR αβ heterodimer can couple to at least two distinct signaling pathways because of the presence of different TCR components. In this model, the small number of receptor complexes that contain CD3-ζη are coupled, directly or indirectly, to the generation of IP and DAG. Most of the TCR complexes, which lack CD3-ζη, are not coupled to this pathway, but can still activate a tyrosine kinase when cross-linked with anti–CD3-ε. We have reported a CD3-ζ–negative 2B4.11 variant, MA 5.8, that expresses one-twentieth of the TCR on its cell surface as the wild type (*7*). Anti–CD3-ε stimulated very little IP from MA 5.8 cells, but the amount was comparable to that generated by another variant that had equally few cell surface TCRs but that synthesized all of the CD3 chains, including ζ. The data obtained with MA 5.8 cells, which cannot express CD3-ζη, and with the CD3-ζη–deficient variants might be resolved in several ways. Given that all of the CD3-ζη variants generate some IP after activation, perhaps some phosphoinositide hydrolysis is independent of CD3-ζη. Alter-

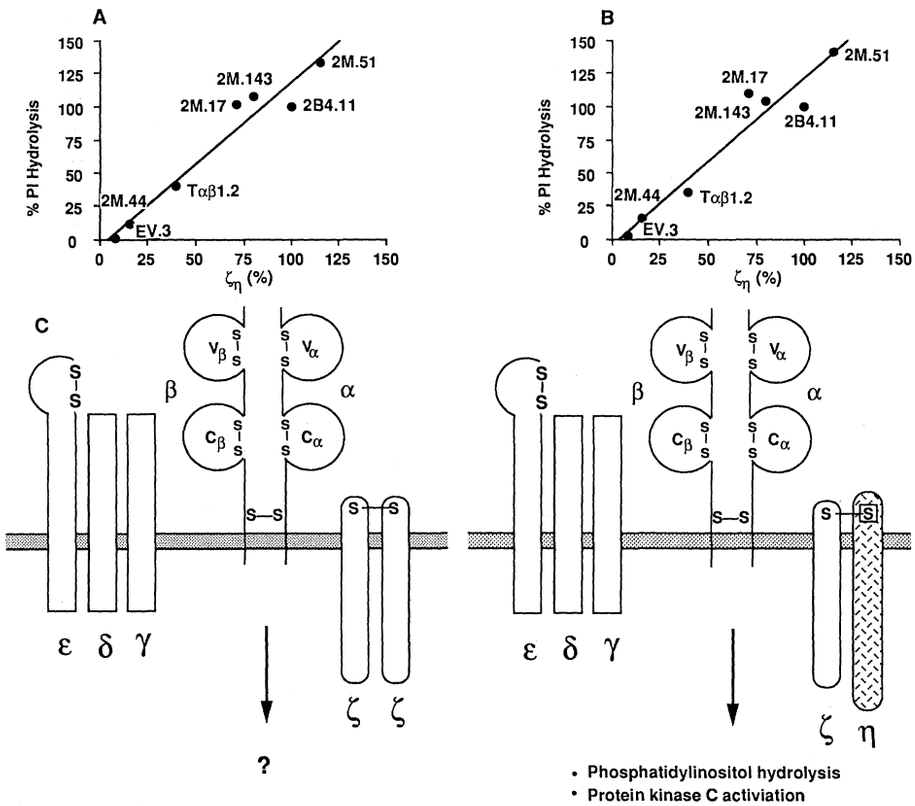

Fig. 5. Correlation between CD3-ζη expression and phosphoinositide hydrolysis. The 2B4.11 cell line and six of its variants were examined for the generation of IP in response to activation with (**A**) pigeon cytochrome c fragment 81-104 or (**B**) anti–CD3-ε, as in Fig. 3. They were independently assessed by immunoblotting for expression of CD3-ζη, as in Fig. 2. The relative amounts of CD3-ζ were reproducible, and remained constant in a given clone over months. Shown is the amount of CD3-ζη plotted as a function of the maximal amount of IP generated over a 90-minute period; the values obtained with 2B4.11 are arbitrarily defined as 100% in both assays. The data were collected from multiple experiments, but 2B4.11 was analyzed in each case. The name of each cloned cell is given next to the corresponding data point. The slopes of the linear regression lines were 1.23 ± 0.14 and 1.25 ± 0.15, respectively, indicating statistical significance ($P < 0.01$). (**C**) Schematic model depicting the correlation between CD3-ζη expression and early biochemical events initiated directly via the TCR. The exact stoichiometry of the subunits, as well as whether a single receptor might contain both ζ_2 and ζη dimers, is unknown.

natively, the presence of the ζ_2 homodimer might prevent the coupling of the TCR to phosphoinositide turnover in complexes containing ζ_2. It should be noted that a dichotomy was found in MA 5.8 between the ability of anti–CD3-ε and antigen to induce IL-2 production. A similar comparison must be made for tyrosine phosphorylation in the new variants to determine if CD3-ζη is important for coupling TCR occupancy to a tyrosine kinase pathway.

Although the generation of IP and the activation of PKC-dependent phosphorylation correlate with the amounts of CD3-ζη, this does not constitute proof that these events are directly related to TCR composition. However, the correlation between the structural and the physiological data does suggest a novel biological mechanism whereby a single ligand-binding molecule can initiate independent intracellular pathways due to an association with different signal transducing elements.

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6. Two (B10.A × AKR)F_1 mice were injected subcutaneously with 2B4.11 T cells and treated with intraperitoneal injections of anti–CD3-ε (2 μg per mouse every other day for 14 days). The tumors that appeared at the site of inoculation in both animals were excised and the cells cultured. One tumor line expressed low levels of TCR, as judged by immunofluorescence staining with MAb A2B4-2 (anti–TCR-α). Cloning by limiting dilution yielded five subclones, all of which stained only weakly with anti–TCR-α and responded poorly (with IL-2 production or inhibition of growth) to stimulation with antigen or anti–CD3-ε. Immunoblots detected little or no CD3-ζ chain on the five clones. The other tumor line was heterogeneous in its expression of anti–TCR-α–reactive material and was cloned twice, yielding 6 and 150 subclones, respectively. Immunoblots with anti–CD3-ζ revealed many of these subclones to be deficient in CD3-ζη (2 of 6 in the first subcloning, 28 of 80 tested in the second subcloning). Of these, two T cell hybridomas from independent subclonings that expressed the lowest levels of CD3-ζη were selected for further analysis: EV.3 and 2M.44.
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17. In experiments (n = 2) to analyze tyrosine phosphorylation of CD3-ζ, unlabeled 2B4.11 and EV.3 cells were stimulated with LK 35.2 cells and a 4% final concentration of anti–CD3-ε supernatant. Antigen receptors were immunopurified as above and subjected to SDS-PAGE. The proteins were transferred to nitrocellulose, and CD3-ζ chains phosphorylated on tyrosine residues were detected with affinity-purified antibodies to phosphotyrosine and ^{125}I-labeled protein A (*14*). To normalize for differences in receptor number between the two cell types, 2B4.11 and EV.3 cells were labeled with ^{32}P and stimulated with doses of PMA that give maximal and stoichiometric CD3-γ phosphorylation (*15*). The level of phosphorylation was determined by densitometry after NEPHGE/SDS-PAGE. Relative tyrosine phosphorylation of CD3-ζ is expressed as the ratio of arbitrary absorbance units: (EV.3ζ/EV.3γ) to (2B4.11ζ/2B4.11γ). In experiments (n = 2) to analyze phosphoinositide hydrolysis, relative receptor density was evaluated by indirect immunofluoresence. Comparison of this technique with densitometric analysis of PMA-induced phosphorylation yielded comparable results when used for normalization. The data represent the arithmetic average of two independent experiments each.
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Limited Immunological Recognition of Critical Malaria Vaccine Candidate Antigens

MICHAEL F. GOOD,* LOUIS H. MILLER, SANJAI KUMAR, ISABELLA A. QUAKYI, DAVID KEISTER, JOHN H. ADAMS, BERNARD MOSS, JAY A. BERZOFSKY, RICHARD CARTER†

Current vaccine development strategies for malaria depend on widespread immunological responsiveness to candidate antigens such as the zygote surface antigens and the sporozoite coat protein, the circumsporozoite (CS) protein. Since immunological responsiveness is controlled mainly by genes mapping within the major histocompatibility complex (MHC), the humoral immune response to the zygote surface antigens and the cytotoxic T lymphocyte (CTL) response to the CS protein were examined in MHC-disparate congenic mouse strains. Only two of six strains responded to the 230-kilodalton zygote surface antigen and another two strains responded to the 48/45-kilodalton surface antigen. From two mouse strains, expressing between them five different class I MHC molecules, there was recognition of only a single CTL epitope from the CS protein, which was from a polymorphic segment of the molecule. The restricted CTL response to this protein parallels the restricted antibody response to this protein observed in humans and mice. These findings suggest that subunit malaria vaccines now being developed may be ineffective.

THE ABILITY OF AN INDIVIDUAL TO respond to a given antigen is governed by immune response (*Ir*) genes and the repertoire of immunoreactive lymphocytes. *Ir* genes, which encode the polymorphic MHC molecules, function by the ability of their gene products to bind T cell antigenic sites derived from the antigen (*1*). *Ir* genes control both CTLs (by class I MHC molecules) and helper T cells. Helper T cells are responsible for helping antibody production by B cells as well as for antibody-independent cellular immunity (by class II MHC molecules). Although there are constraints on which regions of a protein can function as T cell epitopes (*2*), it was expected that most foreign proteins would contain sufficient T epitopes such that they would be recognized by most members of the species. Indeed, foreign proteins such as hepatitis B surface antigen with a monomeric size of 26 kD and staphylococcal nuclease (17 kD) are recognized by most strains of congenic mice that differ in their MHC-encoding region (H-2) (*3*).

Natural immunity to malaria is often slow to develop, and in regions endemic for malaria, children are fully susceptible for a number of years. Most adults in endemic regions are parasitemic because of poor im-

M. F. Good, L. H. Miller, S. Kumar, I. A. Quakyi, D. Keister, J. H. Adams, R. Carter, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892.
B. Moss, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892.
J. A. Berzofsky, Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

*To whom correspondence should be addressed.
†Present address: Department of Genetics, University of Edinburgh, West Mains Road, Edinburgh, Scotland EH9 3JN.