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Differential Susceptibilities of Mice Genomically Deleted of CD4 and CD8 to Infections with *Trypanosoma cruzi* or *Trypanosoma brucei*

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The role of CD4$^+$ and CD8$^+$ T cells in the surveillance of *Trypanosoma cruzi* or *Trypanosoma brucei brucei* was studied in mice which lacked CD4 or CD8 molecules and which were generated by embryonic stem cell technology. Whereas wild-type mice infected with *T. cruzi* (Tulahuén strain) displayed low levels of parasitemia and no mortality, striking increases in parasite growth and mortality occurred in both CD8$^-$ and CD4$^-$ mice. On the contrary, CD8$^-$ and, to a lesser degree, CD4$^-$ mice showed enhanced resistance to *T. b. brucei*. T-cell-dependent immunoglobulin G-specific responses were produced in CD8$^-$ but not CD4$^-$ mice. Normal T-cell proliferative responses were measured in both CD4$^-$ and CD8$^-$ mice. Interleukin-4 production after concanavalin A or anti-CD3 monoclonal antibody stimulation was strikingly enhanced in CD8$^-$ but not CD4$^-$ spleen cells, whereas gamma interferon production was normal in both CD4$^-$ and CD8$^-$ spleen cells. Spleen and lymph node cells from CD8$^-$ (but not CD4$^-$) mice at 20 days postinfection with *T. cruzi* had higher levels of interleukin-4 mRNA than the wild-type controls, as shown in a competitive polymerase chain reaction assay. On the other hand, CD4$^-$ (but not CD8$^-$) mice at 20 days postinfection with *T. cruzi* had lower levels of gamma interferon mRNA than the wild-type mice.

Trypanosomes are microorganisms that can cause disease and death in humans and animals. In Africa, various subspecies of *Trypanosoma brucei* can cause sleeping sickness in humans and severe disease in many animal species. In Latin America, *T. cruzi* infects millions of humans; a significant fraction of them develop heart and/or intestinal disease known as Chagas' disease. Both parasite species have developed different ways of avoiding the immune response of the host. In the case of *T. brucei*, the major surface coat antigen is encoded by a series of variant genes, allowing for the rapid selection of new serological variants during the infection. With regard to *T. cruzi*, the ability of the parasite to infect a wide variety of host cells, thereby hiding from a hostile immune response, is considered a major evasive mechanism.

Here we show that trypanosomes from America or from Africa have developed different strategies regarding CD4- and/or CD8-dependent aspects of the immune response.

MATERIALS AND METHODS

Mice and parasites. Mutant mouse strains without either CD4 or CD8 expression were generated by embryonic stem cell technology (5, 17). CD4$^-$ and CD8$^-$ mice possessed the *H-2^b* haplotype. C57BL/6 mice were used as controls. All mice were between 8 and 12 weeks of age when infected intraperitoneally with 15 *T. cruzi* (Tulahuén strain) or 6×10^5 to 10×10^5 *T. b. brucei* (An Tat 1/1 rodent-adapted strain) organisms obtained from the peripheral blood of infected mice or rats, respectively. Parasitemia was periodically measured in tail vein blood, and mortality was recorded.

Antibody determinations. The contents of anti-*T. cruzi* antibodies in the sera of infected mice were measured by an enzyme-linked immunosorbent assay (ELISA). The plates were coated overnight with 200 μg of whole homogenate from *T. cruzi* epimastigotes per ml. Sera were then added. The plates were subsequently developed with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG) (γ chain specific) (Sigma, St. Louis, Mo.) or anti-mouse IgM (μ chain specific) (Sigma). The assay was standardized between plates by including the titration of serum from a chronically *T. cruzi*-infected mouse.

Cell proliferation and lymphokine quantification assays. Spleen cells from individual mice (3×10^5) were distributed in flat-bottomed microtiter plate wells, and triplicate wells were cultured for 3 days at 37°C in a 5% CO_2 atmosphere in the presence of 5 μg of concanavalin A (ConA) (type IV; Sigma) per ml, 4 μg of anti-murine CD3 monoclonal antibodies (MAbs) per ml, or culture medium. Twenty hours before harvesting, 1 μCi of [^{3}H]thymidine (Amersham, Buckinghamshire, United Kingdom) was added to each well. The supernatants from cell cultures (5×10^6/ml) incubated for 48 h in the presence of ConA or anti-CD3 MAbs were collected. Interleukin-2 (IL-2) activity was measured by culturing a subclone of the IL-2-dependent cell line CTL.L (7) (3×10^4 cells per ml) in the presence of distinct dilutions of supernatants for 24 h at 37°C. Thymidine incorporation was determined after a 4-h pulse. The addition of 1 μg of an anti-IL-2 MAb (S4B6; American Type Culture Collection [ATCC], Rockville, Md.) per ml completely blocked the proliferative response of the CTL.L cells. Up to 50 μg of an anti-IL-4 MAb (11-B-11; ATCC) per ml had no effect on this response.

A capture ELISA was used to measure the presence of gamma interferon (IFN-γ) and IL-4 in the culture superna-

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tants. Anti-murine IFN-γ MAb R-4-A-62 (ATCC) was used as the capture antibody (10 μg/ml), and biotinylated anti-mouse IFN-γ MAb XMG.1 (4 μg/ml) (Pharmigen, San Diego, Calif.) was used for detection. For IL-4 determinations, 10 μg of anti-IL-4 MAb 11-B-11 per ml was used as the capture antibody, and biotinylated anti-IL-4 MAb 24G2 (4 μg/ml) (Pharmigen) was used for detection. Quantification of lymphokines in the supernatants was calculated by comparison with a reference linear regression with known concentrations of recombinant murine γ-IFN or IL-4 (Genzyme, Cambridge, Mass.).

Competitive PCR assay. The accumulation of IL-4 and IFN-γ mRNAs in spleens and lymph nodes was measured in a competitive polymerase chain reaction (PCR) assay (6). RNA from freshly extracted organs was obtained by use of guanidinium acid thiocyanate and phenol-chloroform extraction. Two micrograms of RNA was denatured and reverse transcribed by use of random hexanucleotides ($pd(N)_6$ Pharmacia) as described previously (8). Thereafter, 10 to 50 ng of cDNA was amplified in a 20-μl PCR mixture containing 2 μl of 10× PCR buffer (Boehringer), 3 μl of deoxynucleoside triphosphates (0.125 mM each), 0.1 μl of *Taq* polymerase (5 U/ml) (Boehringer), 7.5 μl of cDNA, and 2 μl of a competitor fragment with a different length but with the same primers as the target DNA. The IFN-γ competitor was constructed by ligating an unrelated DNA fragment (188 bp) into a *Pst* site of the subcloned PCR product. The β-actin competitor was built by deleting an *Mse*I fragment from a plasmid containing the whole cDNA sequence. The IL-4 competitor was purchased from Clonetech (Palo Alto, Calif.). Competitors were amplified by PCR, purified (PCR Purification Kit; Qiagen, Studio City, Calif.), and quantified in a spectrophotometer.

The primer sequences for the amplification of the cDNA were as follows: sense IFN-γ, 5′ AAC GCT ACA CAC TGC ATC TTG G 3′; antisense IFN-γ, 5′ GAC TTC AAA GAG TCT GAG G 3′; sense IL-4, 5′ ATG GGT CTC AAC CCC CAG CTA 3′; antisense IL-4, 5′ GCT CTT TAG GCT TTC CAG GAA GTC 3′; sense β-actin, 5′ GTG GGC CGC TCT AGG CAC CAA 3′; and antisense β-actin, 5′ CTC TTT GAT GTC ACG CAC GAT TTC 3′.

Ten or 3-fold serial dilutions of the competitor were amplified in the presence of a constant amount of cDNA. Reactions were carried out for 28 to 45 cycles in a thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) with an annealing step at 55°C (for IFN-γ) or 60°C (for IL-4 or β-actin). The PCR products were then resolved by 1.8% agarose gel electrophoresis and photographed. The negative of the photograph was analyzed by laser densitometry, and the absolute values of the PCR products were determined. The ratios of the absorbances of the relevant PCR product pairs were plotted against the concentration of the competitor DNA used. The intersection of the curves at which the amounts of target and competitor were equal was extrapolated to the *x* axis (concentration of the competitor added).

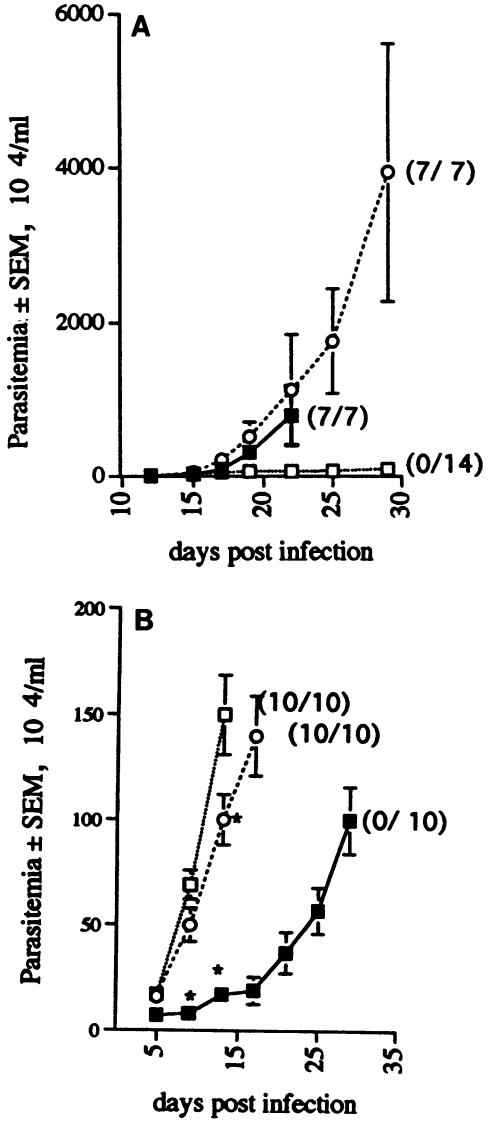

FIG. 1. Parasitemia and mortality of $CD4^-$ $CD8^+$ (○), $CD4^+$ $CD8^-$ (■), and $CD4^+$ $CD8^+$ (□) mice infected intraperitoneally with 15 *T. cruzi* (A) or 6×10^5 to 10×10^5 *T. b. brucei* (B) organisms. The mean number of parasites per milliliter ± the standard error of the mean (SEM) for one of two representative experiments with each parasite species is depicted. The cumulative mortality at 30 (A) or 24 (B) days postinfection is indicated in parentheses. Similar results were obtained when $CD8^-$ ($H\text{-}2^d$) mice were infected and DBA/2 mice were used as controls (data not shown). *, Differences versus wild-type controls were significant ($P < 0.05$; Mann-Whitney–Wilcoxon test). Differences in parasitemia between *T. cruzi*-infected mutant and wild-type mice were significant at 15, 17, 19, 22, 25, and 29 days postinfection ($P < 0.01$; Mann-Whitney U–Wilcoxon test).

RESULTS AND DISCUSSION

Gene knockout mice provide clean experimental models for studying null mutations of specific genes, clearing up controversial points in experiments in which the complete absence of gene activity, achieved by depletion methods, is called into question.

Mice selectively lacking $CD4^+$ or $CD8^+$ T cells (obtained by homologous recombination in embryonic stem cells [5, 17]) and wild-type mice were inoculated with *T. b. brucei* or a reticulotropic strain (Tulahuén) of *T. cruzi*. In the first set of experiments, *T. cruzi* bloodstream-form trypomastigotes were inoculated into $CD8^-$, $CD4^-$ or wild-type mice. Whereas wild-type mice displayed low levels of parasitemia, striking increases in bloodstream-form levels occurred in both $CD8^-$ and $CD4^-$ mice (Fig. 1A). All mutant mice died, whereas all control mice survived.

In the second set of experiments, *T. b. brucei* was likewise inoculated into $CD4^-$, $CD8^-$, or wild-type mice (Fig. 1B). In dramatic contrast to the results obtained with *T. cruzi*, the

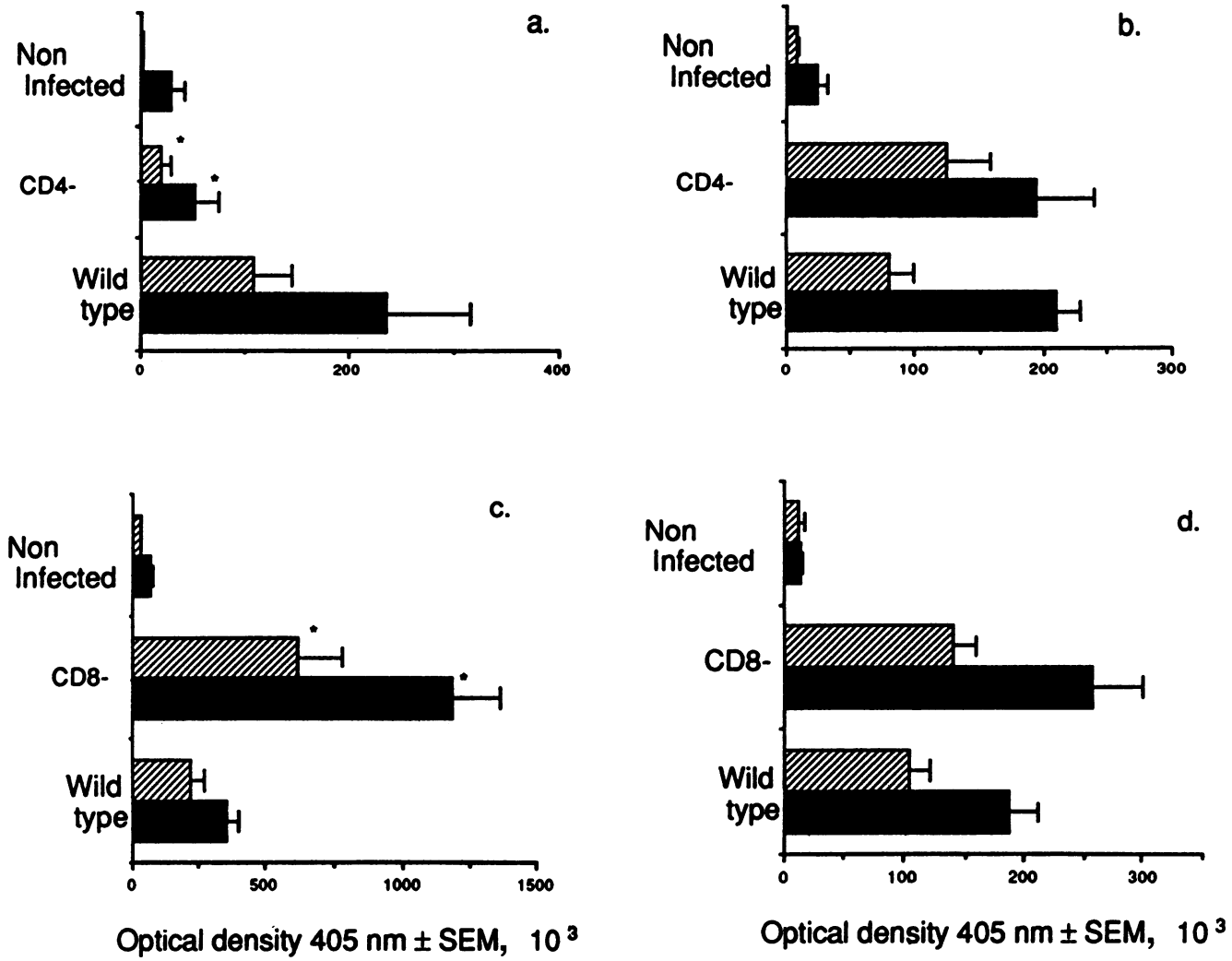

FIG. 2. Generation of IgM (b and d) and IgG (a and c) anti-*T. cruzi* antibodies at 20 days postinfection with *T. cruzi* by CD4$^-$ (a and b), CD8$^-$ (c and d), and control mice (a, b, c, and d). The relative concentrations of specific antibodies in 1/100 (■) or 1/300 (▨) dilutions of sera from individual mice (8 to 10 per group) were determined by an ELISA. The mean absorbance ± the standard error of the mean (SEM) for one of two experiments is depicted. *, Differences versus wild-type infected controls were significant ($P < 0.05$; Student t test).

CD8$^-$ mice were much more resistant to *T. b. brucei* infection. In CD8$^-$ mice, the development of parasitemia was suppressed and survival was twice as long as that in the control mice, reproducing recently published data (14). CD4$^-$ mice were also infected with *T. b. brucei*. In such mice, resistance to *T. b. brucei* was enhanced (Fig. 1B).

The fact that CD4$^-$ and CD8$^-$ mice are dramatically susceptible to *T. cruzi* infection is in line with data reporting T cells to be one of the major cell populations involved in providing resistance to this parasite (9, 11, 22, 23). However, the opposite findings obtained during *T. b. brucei* infection suggest that CD8$^+$ and, to a lesser extent, CD4$^+$ T-cell responses may promote parasite growth. Data obtained from previous studies in less succinct experimental systems support the above-described findings. The partial elimination of CD4$^+$ or CD8$^+$ T cells in vivo by the administration of MAbs enhances susceptibility to *T. cruzi* (1, 20, 21, 26). Likewise, β_2-microglobulin-deficient mice, which are largely devoid of the CD8$^+$ T-cell subset, suffer high mortality when infected with *T. cruzi* (27). In contrast, inoculation of anti-CD8 MAbs into rats makes the animals more resistant to *T. b. brucei* infection (3).

T-cell-dependent IgG responses are produced (and enhanced) in CD8$^-$ but not CD4$^-$ mice (Fig. 2), whereas the induction of cytotoxic T cells is noted in CD4$^-$ mice only (5, 17). We explored the capacity of CD4$^-$ or CD8$^-$ spleen cells from noninfected mice to respond by proliferation or cytokine production to various stimuli (Table 1). Normal T-cell proliferative responses were measured in both CD4$^-$ and CD8$^-$ mutant mice. IL-2 production was enhanced in CD8$^-$ and reduced in CD4$^-$ spleen cells, whereas IFN-γ production was normal in both CD4$^-$ and CD8$^-$ spleen cells. IL-4 production was strikingly enhanced in CD8$^-$ mice compared with controls after ConA or anti-CD3 MAb stimulation both at the protein (Table 1) and at the mRNA (data not shown) levels. Spleen and lymph nodes from CD8$^-$ mice at 20 days postinfection with *T. cruzi* contained a significantly higher level of IL-4 mRNA than the wild-type controls, as measured in a quantitative PCR assay (Table 2). Thus, the enhanced susceptibility of CD8$^-$ mice to *T. cruzi* is associ-

TABLE 1. Proliferative responses of and lymphokine production by CD4$^-$ and CD8$^-$ mutant mice exposed to ConA or anti-CD3 MAbs[a]

Group	Stimulus	Proliferation (cpm)	IL-2 (cpm)	IFN-γ (ng/ml)	IL-4 (pg/ml)
Wild type	ConA	288,500 ± 51,136	44,800 ± 8,100	85 ± 9	24 ± 12
CD8$^-$	ConA	330,900 ± 23,520	75,700 ± 4,000[b]	102 ± 27	328 ± 80[b]
Wild type	Anti-CD3 MAbs	328,700 ± 28,426	14,600 ± 4,200	52 ± 20	132 ± 31
CD8$^-$	Anti-CD3 MAbs	354,700 ± 19,317	40,800 ± 4,100[b]	54 ± 13	749 ± 223[b]
Wild type	ConA	212,700 ± 23,637	72,100 ± 18,700	38 ± 14	23 ± 12
CD4$^-$	ConA	310,100 ± 17,176	14,300 ± 3,600[b]	51 ± 11	58 ± 21
Wild type	Anti-CD3 MAbs	244,200 ± 12,399	22,600 ± 2,800	88 ± 16	32 ± 3
CD4$^-$	Anti-CD3 MAbs	325,750 ± 11,161	5,700 ± 2,500[b]	80 ± 19	130 ± 51

[a] The mean ± the standard error of the mean for the proliferative responses or the lymphokine content in culture supernatants from spleen cells from four uninfected mice per group is shown. The data are representative of three independent experiments.
[b] Differences from the wild-type group were significant ($P < 0.05$; Student t test).

ated with enhanced production of IL-4. Interestingly, IL-4 is able to down-regulate the parasiticidal mechanisms of macrophages (24) and plays a role in the susceptibility of BALB/c mice to *Leishmania major* (10). When treated with anti-IL-4 MAbs, *T. cruzi*-infected mice showed a lower parasite load (15).

An important difference in the life cycles of *T. cruzi* and *T. brucei* is the site of growth. *T. brucei* is an extracellular parasite, whereas *T. cruzi* multiplies in the cytoplasm when infecting the mammalian host. *T. brucei* would thus be considered to be explicitly under attack by humoral antibodies. *T. cruzi*, on the other hand, would, because of its growth habit, be considered a target for cytotoxic cells, be they of T-cell or natural killer cell nature. The release of trypomastigotes into the bloodstream also allows T-cell-dependent antibodies to play a role in blocking the in vivo spread of *T. cruzi*. Specific antibodies neutralize, lyse, transfer resistance against *T. cruzi*, and participate in vitro in cellular effector mechanisms of parasite destruction (4). On the other hand, the slightly enhanced resistance of CD4$^-$ mice to *T. brucei* argues against an involvement of T-cell-dependent antibodies, at least in this particular combination of mouse and parasite strains. In accordance, mice infected with *T. brucei* generate antibodies to the major surface coat antigen and control parasitemia in the absence of T-cell help (19). The reason that both CD4$^-$ and CD8$^-$ mice become strikingly susceptible to *T. cruzi* infection may thus be manifold and involve both humoral and cellular factors. The behavior of CD8$^-$ mice in displaying enhanced resistance to *T. brucei* is likely to involve the unusual capacity of this parasite to release a molecule which triggers IFN-γ production by T cells by binding to the CD8 molecule (2, 14). This cytokine behaves like a significant growth factor for *T. brucei* (13). The growth-promoting role of IFN-γ for *T. brucei* contrasts with that for *T. cruzi*, for which IFN-γ is involved in resistance to the parasite, probably through the activation of trypanocidal mechanisms of phagocytes (12, 16, 18, 25, 28). CD4$^-$ (but not CD8$^-$) mice at 20 days postinfection with *T. cruzi* showed lower levels of IFN-γ mRNA than the wild-type controls (Table 2).

In conclusion, although belonging to the same genus, *T. cruzi* and *T. brucei* show differences in pathophysiology in their hosts which are clearly reflected by their interaction with the T-cell compartment of the immune system.

TABLE 2. IL-4 and IFN-γ mRNA levels in spleen and lymph node cells from CD4$^-$, CD8$^-$, or wild-type mice at 20 days postinfection with *T. cruzi*[a]

Group	Mol of IL-4/mol of β-actin (10^4) in:		Mol of IFN-γ/mol of β-actin (10^3) in:	
	Spleen	Lymph nodes	Spleen	Lymph nodes
CD8$^-$	1.63[b]	0.91[b]	68	124
Wild type	<0.32	<0.09	85	116
CD4$^-$	0.041	<0.02	19[b]	30[b]
Wild type	0.044	<0.03	112	87

[a] Total RNAs from spleens or lymph nodes of individual mice were transcribed into cDNAs. Aliquots of cDNA were amplified with β-actin, IFN-γ, or IL-4 primers in the presence of threefold serial dilutions of the respective competitors. The mean moles of IFN-γ or IL-4 mRNA per mol of β-actin mRNA from three samples per group are shown. The data are representative of three independent experiments.
[b] Differences from the wild-type controls were significant ($P < 0.05$; Student t test).

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