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Compromised Influenza Virus-Specific CD8$^+$-T-Cell Memory in CD4$^+$-T-Cell-Deficient Mice

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The primary influenza A virus-specific CD8$^+$-T-cell responses measured by tetramer staining of spleen, lymph node, and bronchoalveolar lavage (BAL) lymphocyte populations were similar in magnitude for conventional *I-A*$^{b+/+}$ and CD4$^+$-T-cell-deficient *I-A*$^{b-/-}$ mice. Comparable levels of virus-specific cytotoxic-T-lymphocyte activity were detected in the inflammatory exudate recovered by BAL following challenge. However, both the size of the memory T-cell pool and the magnitude of the recall response in the lymphoid tissues (but not the BAL specimens) were significantly diminished in mice lacking the CD4$^+$ subset. Also, the rate of virus elimination from the infected respiratory tract slowed at low virus loads following challenge of naïve and previously immunized *I-A*$^{b-/-}$ mice. Thus, though the capacity to mediate the CD8$^+$-T-cell effector function is broadly preserved in the absence of concurrent CD4$^+$-T-cell help, both the maintenance and recall of memory are compromised and the clearance of residual virus is delayed. These findings are consistent with mathematical models that predict virus-host dynamics in this, and other, models of infection.

The extent to which virus-specific CD8$^+$-T-cell responses are dependent on concurrent help from CD4$^+$ T cells has long been a matter of debate (12, 25). The nature of the discussion has evolved with the development of improved assay systems (3, 27), with the extension of the original observations to a greater range of virus models, and with the realization that CD4$^+$ T cells function to activate dendritic cells (7, 23, 32, 33). The net consequence of this activation is that the molecular context for effective antigen presentation to the responding CD8$^+$ T lymphocytes is enhanced. Some of the differences in ideas about the perceived requirement for CD4$^+$-T-cell help for CD8$^+$-T-cell responses may, in fact, reflect the degree to which a particular pathogen directly activates dendritic cells.

According to the present stage of understanding, CD8$^+$ T cells do not deal well with high-level, persistent virus infections in the absence of an ongoing CD4$^+$-T-cell response (21, 22). This is at least part of the reason why human immunodeficiency virus infections that are not treated with drugs progress to AIDS. Experimentally, the consequences of CD4$^+$-T-cell deficiency have been shown most clearly with the lymphocytic choriomeningitis virus (LCMV) model (10, 34). Mice with a disrupted *I-A*^{b} major histocompatibility complex (MHC) class II glycoprotein (*I-A*$^{b-/-}$ mice) can maintain normal numbers of LCMV-specific CD8$^+$ T cells in the presence of persistent virus, but the lymphocytes progressively lose the capacity to make effector cytokines (42) like gamma interferon (IFN-γ). The LCMV model is fairly unusual, as the virus is not lytic and mice can tolerate massive levels of infection in the absence of clinical impairment.

The situation is quite different for infections with lytic murine gammaherpesvirus 68 (γHV68), with which *I-A*$^{b-/-}$ mice eventually die as a consequence of continuing, but relatively low-level, infection (9). The capacity of γHV68-specific CD8$^+$ T cells to make IFN-γ is preserved up to the stage at which *I-A*$^{b-/-}$ mice exhibit symptoms of wasting disease (4). The key defect in this experimental system may be the absence of IFN-γ-producing CD4$^+$ effectors that mediate direct control of the infection (11). Even so, some evidence of diminished activation and a reduced capacity to make IFN-γ is found (24) when the numbers of responding CD8$^+$ T cells in *I-A*$^{b-/-}$ mice are massively expanded by a postexposure vaccination protocol (4).

The influenza A viruses are also lytic but (unlike γHV68) have no capacity to establish latency and are thought to be completely eliminated from *I-A*$^{b-/-}$ mice by the antigen-specific CD8$^+$-T-cell response (13, 38). Though influenza virus infection can be controlled in the absence of CD8$^+$ T cells, such MHC class I-glycoprotein-deficient, β2-microglobulin-negative mice are much more likely to succumb following exposure to highly pathogenic viruses (14, 15). The protective effect of CD4$^+$ T cells is believed to operate via the provision of help for the virus-specific antibody response (26, 35, 37). Mice that lack both CD8$^+$ and CD4$^+$ T cells invariably die following respiratory challenge with even a mild influenza virus strain (1).

The initial study of influenza virus infection in *I-A*$^{b-/-}$ mice led to the conclusion that, despite a slight delay in virus clearance from the lung, the CD8$^+$-T-cell response was not significantly compromised (8). Further experiments confirmed this pattern of slower virus elimination and showed that the levels of virus-specific cytotoxic T-lymphocyte (CTL) activity were comparable for bronchoalveolar lavage (BAL) specimen pop-

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ulations recovered from the respiratory tracts of $I\text{-}A^{b+/+}$ and $I\text{-}A^{b-/-}$ mice (38). However, limiting dilution analysis of CTL precursor (CTLp) frequencies indicated that the extent of CTLp expansion was substantially diminished following both primary and secondary challenges in the absence of a concurrent $CD4^+$-T-cell response. The conclusion was that the host used the available CTLp pool to develop the level of CTL effector function necessary to control the pathogen and that the remaining CTLp contributed to the establishment of a reduced pool of memory T cells (relative to that of the $I\text{-}A^{b+/+}$ controls) (38). The present experiments analyze this question again by measuring the influenza virus-specific $CD8^+$-T-cell response (5, 16) with tetrameric complexes of MHC class I glycoprotein plus peptide (tetramers).

Experimental procedures. The methods used here have all been described in detail previously (2, 6, 16) The female $I\text{-}A^{b+/+}$ C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Maine), while the congenic $I\text{-}A^{b-/-}$ mice (18, 38) were bred at St. Jude Children's Research Hospital. Adult (6- to 8-week-old) mice were anesthetized and infected intranasally (i.n.) with $10^{6.8}$ 50% egg infective doses (EID_{50}) of the HKx31 (H3N2) influenza A virus. Secondary responses were generated by i.n. HKx31 challenge of mice primed intraperitoneally (i.p.) with $10^{8.5}$ EID_{50} of the A/PR/8/34 (PR8, H1N1) virus at least 6 weeks earlier. At the time of sampling, the lungs were removed and virus titers were determined by the inoculation of clarified tissue homogenates into embryonated chicken eggs.

The spleens and mediastinal lymph nodes (MLN) were disrupted and $CD8^+$ T cells were enriched by incubation with monoclonal antibodies to CD4 (GK1.5) and the MHC class II glycoprotein (M5/114.15.2) and then with anti-rat and anti-mouse immunoglobulin G (IgG)-coated magnetic beads (Dynal A.S., Oslo, Norway). Lymphocytes were obtained from pneumonic lungs by BAL, and macrophages were removed by incubation on plastic for 1 h at 37°C. Levels of CTL activity were determined using a conventional, 6-h ^{51}Cr release assay. The D^bNP_{366} (16) and D^bPA_{224} (5) tetramers were made by complexing $H2D^b$ with the influenza virus nucleoprotein (NP) ASNENMETM (for D^bNP_{366}) or the acid polymerase protein (PA) SSLENFRAYV (for D^bPA_{224}). Lymphocytes were incubated for 60 min at room temperature with the phycoerythrin-conjugated tetramers in phosphate-buffered saline–bovine serum albumin–azide, followed by staining with anti-CD8α antibody–fluorescein isothiocyanate (PharMingen, San Diego, Calif.). Other $CD8^+$ T cells were incubated with 1 μM peptide for 5 h in the presence of 5 μg of Brefeldin A (Epicentre Technologies, Madison, Wis.)/ml, fixed, and stained for CD8α antibody and IFN-γ (phycoerythrin-XMG 1.2). All data were acquired with a Becton Dickinson FACScan and then analyzed with CELLQuest software (Becton Dickinson Immunocytometry Systems, San Jose, Calif.).

Mathematical modeling. The data set from these experiments was also used to model influenza virus infection in normal and $CD4^+$-T-cell-deficient mice. We considered a very basic model of infection dynamics involving three variables: uninfected susceptible host cells (x), infected host cells (y), and a CTL response (z). The mathematical model is given by the following set of differential equations (28, 29): $x = \lambda - dx - \beta xy$, $y = \beta xy - ay - pyz$, and $z = cy - bz$. Susceptible host cells are produced at rate λ, die at rate dx, and become infected by the virus at rate βxy. The infected cells die at rate ay and are killed by CTLs at rate pyz. The CTLs expand in response to the antigen at rate cy and decay at rate bz in the absence of antigenic stimulation. Note that this is one of the simplest ways to model CTL expansion and dynamics. CTL expansion can be described in several different ways, but the arguments made in the present context remain robust as long as CTL expansion depends on the level of antigen (41). The CTL population, z, can be viewed as an effector response. More-complex models distinguishing between memory and effector CTLs have been published (40). For a detailed analysis of the model presented here, see the work of Wodarz et al. (41).

The present application simulates the dynamics of influenza virus infection in $I\text{-}A^{b+/+}$ and $I\text{-}A^{b-/-}$ mice and quantifies differences in the efficacies of the immune responses from rates of virus decline during the resolution phase of the disease process. The virus population decays if the rate of virus elimination is greater than the rate of virus replication [$(a + pz) > \beta x$]. The rate of change of the virus population is given by the equation $\beta x - a - pz$. When the virus load is around its peak, the number of susceptible cells will be relatively low, and hence βx will be relatively small. Virus load thus decays at a rate which can be approximated by the equation $a + pz$. Hence, the rate of virus decline is proportional to the strength of the CTL response. The strength of the CTL response is determined by three parameters in the model: the rate of CTL expansion, c; the rate of CTL decline, b; and the rate of CTL-mediated effector activity, p.

Virus clearance and CTL effector function. The earlier finding (38) that virus clearance was delayed in both primarily and secondarily challenged $I\text{-}A^{b-/-}$ mice was confirmed (Fig. 1) and refined by mathematical analysis. The rate of virus elimination from the lungs of the control $I\text{-}A^{b+/+}$ mice showed a clear pattern of exponential decay, with exponents of virus decay of 3.4 per day after the primary challenge and 2.7 per day after the secondary challenge. This was also the case initially for the $I\text{-}A^{b-/-}$ mice, which showed exponents of virus decay of 2.3 per day after the primary challenge and 1.5 per day after the secondary challenge. However, as the virus load was reduced, the rate of virus decline slowed significantly. Despite this difference in the virus "tails" (Fig. 1) for the $I\text{-}A^{b+/+}$ and $I\text{-}A^{b-/-}$ mice, the levels of lytic activity in the BAL specimen populations recovered from secondarily challenged mice were (as shown previously) generally comparable (Fig. 2).

Quantitation of the $CD8^+$-T-cell response. Measurement of the primary $CD8^+$-T-cell response by staining with the D^bNP_{366} and D^bPA_{224} tetramers showed little difference in magnitudes for the $I\text{-}A^{b+/+}$ and $I\text{-}A^{b-/-}$ mice (Fig. 3). However, the situation changed dramatically following secondary challenge. The numbers of $D^bNP_{366}{}^+$ and $D^bPA_{224}{}^+$ $CD8^+$ T cells generated in the spleens and MLN of the $I\text{-}A^{b-/-}$ mice were substantially lower than those in the $I\text{-}A^{b+/+}$ controls (compare panels A and B and panels C and D of Fig. 4), though the values for the BAL specimens were comparable (Fig. 4E and F). The patterns for the spleens were confirmed in a further experiment, where the differences for both epitopes were significant at every time point (Table 1). The defect in the $I\text{-}A^{b-/-}$ mice was also apparent in memory $CD8^+$-T-cell populations sampled immediately prior to virus

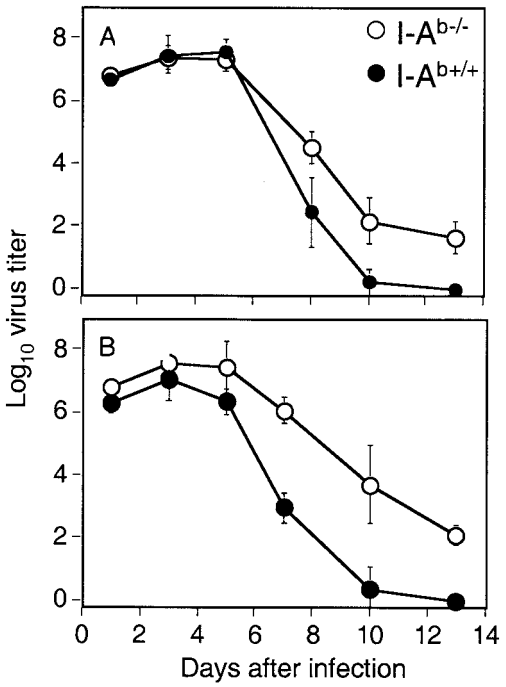

FIG. 1. Lung virus titers following primary (A) or secondary (B) challenge of *I-A*$^{b+/+}$ and *I-A*$^{b-/-}$ mice. Naïve (A) or PR8-primed ($10^{8.5}$ EID_{50} i.p. 6 weeks earlier) (B) mice were given $10^{6.8}$ EID_{50} i.n. and sampled at the intervals shown. Log_{10} dilutions of lung homogenate were used to infect embryonated chicken eggs, and endpoint titrations were determined by a hemagglutination inhibition assay of allantoic fluid. The data are expressed as means ± standard deviations (SD) for groups of five mice each. No virus was recovered from five naïve *I-A*$^{b-/-}$ mice sampled at 19 days after infection with the HKx31 virus strain.

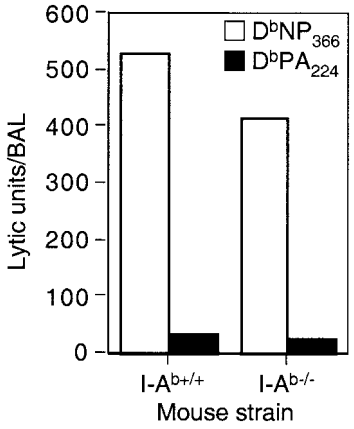

FIG. 2. Peptide-specific CTL activity in the inflammatory cell population recovered by BAL of the infected respiratory tract following secondary i.n. challenge with the HK31 influenza A virus 8 days earlier (see the legend for Fig. 1). The EL4 ($H2^b$) target cells were pulsed with either the NP_{366} or the PA_{224} peptide prior to use in a standard 6-h ^{51}Cr release assay. Lytic activities were calculated for the $CD8^+$ $D^bNP_{366}{}^+$ and $CD8^+$ $D^bPA_{224}{}^+$ tetramers as determined by tetramer staining (data not shown).

challenge (day 0) (Table 1). However, though the numbers were lower throughout the response in the *I-A*$^{b-/-}$ mice, the capacity of these T cells to make IFN-γ after short-term in vitro stimulation with peptide was not obviously diminished (Table 1).

Overall, these results indicate that, though the primary response was not numerically compromised in the *I-A*$^{b-/-}$ mice, the capacity to generate an effective $CD8^+$-memory T-cell pool was diminished. Recent experiments have suggested that the early triggering of naïve $CD8^+$ T cells drives the responding lymphocytes through a full program of division without any requirement for additional encounters with the antigen (19, 39). Perhaps, however, this is not equally true for the differentiation of these lymphocytes. Though the D^bNP_{366}-specific and D^bPA_{224}-specific primary responses were comparable for the *I-A*$^{b+/+}$ and *I-A*$^{b-/-}$ mice (Fig. 3), the frequencies determined previously (38) from limiting dilution analysis microcultures were much lower for the *I-A*$^{b-/-}$ group, the size of the memory T-cell pool measured by tetramer staining (Table 1) was reduced, and the remaining memory T cells generated a diminished secondary response (Fig. 4). The earlier finding that the extents of CTL effector function in the lung are comparable for influenza virus-infected *I-A*$^{b+/+}$ and *I-A*$^{b-/-}$ mice was also confirmed and extended by the demonstration that the numbers of $D^bNP_{366}{}^+$ and $D^bPA_{224}{}^+$ $CD8^+$ T cells recovered by BAL were equivalent.

$CD4^+$-T-cell help for B cells and $CD8^+$ T cells. The protracted clearance of the virus tail in the *I-A*$^{b-/-}$ mice (Fig. 1) was not reflected in any obvious (Fig. 2; Table 1) defect in the level of CTL effector function (in the BAL specimens) or in the capacity to produce IFN-γ following short-term, in vitro stimulation with peptide (in the spleens). However, many other parameters that were not measured may potentially have modified the capacity of these T cells to function. These parameters include the production of other cytokines and chemokines and the expression of various adhesion molecules that might help to guide the immune T cell to the increasingly rare (as the virus is eliminated) infected target (6, 10, 13, 24).

The broad alternative to the idea that $CD4^+$-T-cell involvement is necessary to produce optimal CTL effectors in this experimental model of lytic infection is that the clean-up of residual virus is more efficiently handled by the CD4-dependent antibody response (30). The virus tail effect described here for the *I-A*$^{b-/-}$ mice can also be seen in $Ig^{-/-}$ μMT mice (20), which clear influenza viruses more slowly than conventional $Ig^{+/+}$ *I-A*$^{b+/+}$ controls (17, 35). This is likely to be a consequence of the lack of antibody. However, it is also apparent that $CD4^+$ T cells contribute substantially to the recovery of virus-infected μMT mice. Depleting $CD4^+$ T cells by treatment with monoclonal antibodies made these $Ig^{-/-}$ mice much more susceptible to infection with the HKx31 influenza A virus (31). The magnitude of the $CD8^+$-T-cell response was diminished, though the effect was less consistent than that found by the present study in *I-A*$^{b-/-}$ mice. Unlike the *I-A*$^{b-/-}$ mice, the μMT animals have minute spleens and disrupted lymphoid architecture due to the complete absence of both B cells and follicular dendritic cells. Though they can make effective influenza virus-specific $CD4^+$- and $CD8^+$-T cell-responses and develop long-term memory in both compartments (31, 36), the magnitudes of these populations are (due to the small spleen size) diminished and the responses are often more variable. Overall, the results from both the *I-A*$^{b-/-}$ mice (Ta-

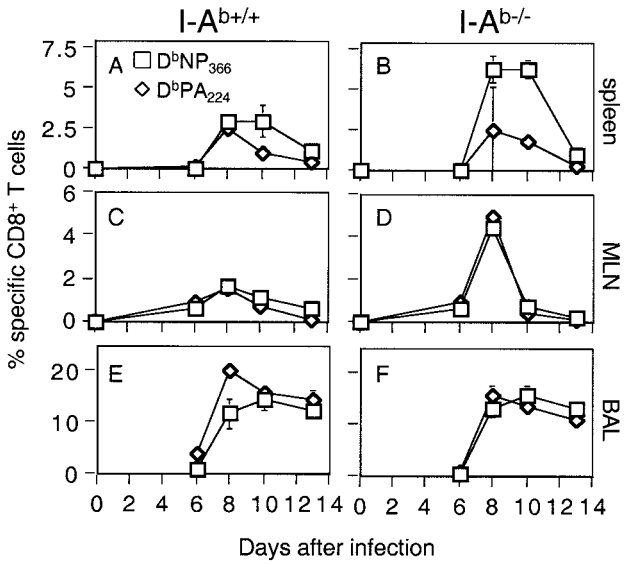

FIG. 3. Kinetic analysis of the D^bNP_{366} and D^bPA_{224} epitope-specific $CD8^+$-T-cell response in $I\text{-}A^{b+/+}$ and $I\text{-}A^{b-/-}$ mice following primary i.n. challenge with the HKx31 influenza virus. Enriched spleen (A and B), MLN (C and D), and BAL (E and F) samples were stained with anti-CD8 antibody and either the D^bNP_{366} or the D^bPA_{224} tetramer. Each data point represents the mean ± SD of values for the spleen samples and pooled MLN and BAL samples for five mice. Similar results were obtained from two further experiments.

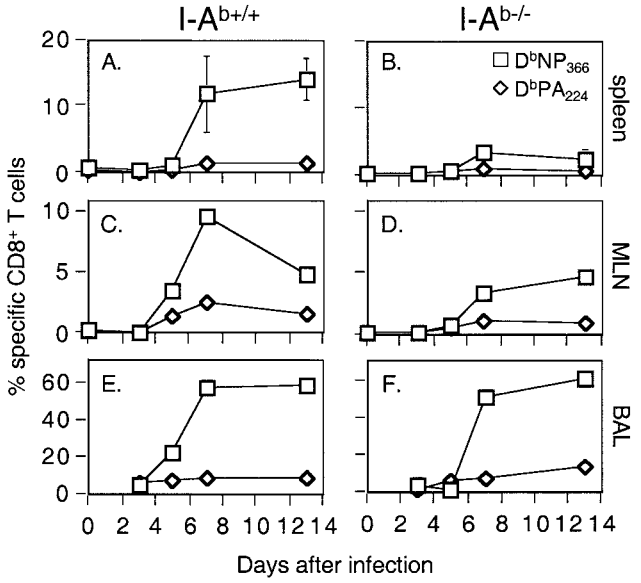

FIG. 4. Secondary D^bNP_{366} and D^bPA_{224} epitope-specific $CD8^+$-T-cell responses in the spleen (A and B), MLN (C and D), and BAL (E and F) samples from $I\text{-}A^{b+/+}$ (A, C, and E) and $I\text{-}A^{b-/-}$ (B, D, and F) mice. The mice had been primed i.p. with the PR8 virus 6 weeks before i.n. challenge with the HKx31 virus. Each data point represents the mean ± SD of values for the spleen samples and pooled MLN and BAL samples for five mice. Similar results were obtained from two further experiments.

ble 1; Fig. 4) and the $Ig^{-/-}$ mice (31) indicate that the development of optimal virus-specific effector CTLs and $CD8^+$ memory T cells in this localized, limited virus infection requires concurrent help from $CD4^+$ T cells.

Application of the mathematical model. The model estimates the rate of virus elimination during the resolution phase of the infection. Following primary or secondary challenge, the rate of virus decline was faster in wild-type than in $CD4^+$-T-cell-deficient mice. Clearly, the immune response was less efficient in the absence of the $CD4^+$-T-cell subset.

Simulations of the infection dynamics in wild-type and $CD4^+$-T-cell-deficient hosts are illustrated in Fig. 5. In accordance with the experimental data, the assumption is that help from $CD4^+$ T cells can influence two parameters of the CTL response: the rate of CTL expansion (c) and the life span of the CTLs ($1/b$). It is assumed that $CD4^+$-T-cell-deficient hosts are characterized by a lower rate of CTL expansion (lower value of c) or a shorter life span of the CTLs (lower value of $1/b$). Experimental simulations to test both assumptions result in similar dynamics (Fig. 5). The resolution of the infection is delayed in the absence of concurrent $CD4^+$-T-cell help. At the same time, the numbers of CTLs (which should be considered effectors in the model) follow similar time courses in both cases. The reason is as follows. If the CTL response is stronger, the infection is resolved more quickly, resulting in a lower virus

TABLE 1. Comparison of the $CD8^+$-T-cell responses by tetramer and IFN-γ staining[a]

Tetramer	No. of days after challenge with HKx31	% of epitope-specific $CD8^+$ T cells in spleens of[b]:			
		$I\text{-}A^{b+/+}$ mice		$I\text{-}A^{b-/-}$ mice	
		Tetramer staining	IFN-γ staining	Tetramer staining	IFN-γ staining
D^bNP_{366}	0	1 ± 0.24 ($P < 0.02$)	0.4 ± 0.2	0.6 ± 0.14	0.2 ± 0.1
	5	1.4 ± 0.4 ($P < 0.0025$)	1.5 ± 0.7	0.1 ± 0.1	0.75 ± 1.4
	7	20.4 ± 5.9 ($P < 0.0025$)	13.6 ± 4.3	3.2 ± 2.0	1.8 ± 1.3
	10	24.1 ± 11.4 ($P < 0.01$)	21.5 ± 9.6	8.3 ± 2.8	7.1 ± 2.2
D^bPA_{224}	0	0.5 ± 0.1 ($P < 0.02$)	0.2	0.3 ± 0.1	0.1 ± 0.1
	5	0.3	0.2	0.04	0.04
	7	2 ± 0.6 ($P < 0.0025$)	1.4 ± 0.6	0.4 ± 0.1	0.1 ± 0.1
	10	2.5 ± 0.8 ($P < 0.05$)	1.9 ± 0.6	1.3 ± 0.8	0.7 ± 0.6

[a] The $I\text{-}A^{b+/+}$ and $I\text{-}A^{b-/-}$ mice were primed i.p. with PR8 virus and challenged i.n. with the HKx31 virus 56 days later as described in the legend for Fig. 1. The epitope-specific $CD8^+$ T cells were identified by tetramer staining or by short-term (5-h) stimulation with peptide in the presence of Brefeldin A.

[b] Groups of five mice were analyzed at each time point. The results are expressed as mean percentages of $CD8^+$ T cells that were specific for the $D^bNP_{366}{}^+$ or $D^bPA_{224}{}^+$ epitope ± SD, and values in parentheses reflect the results of a statistical analysis comparing the values for the tetramer-positive cells in $I\text{-}A^{b+/+}$ and $I\text{-}A^{b-/-}$ mice.

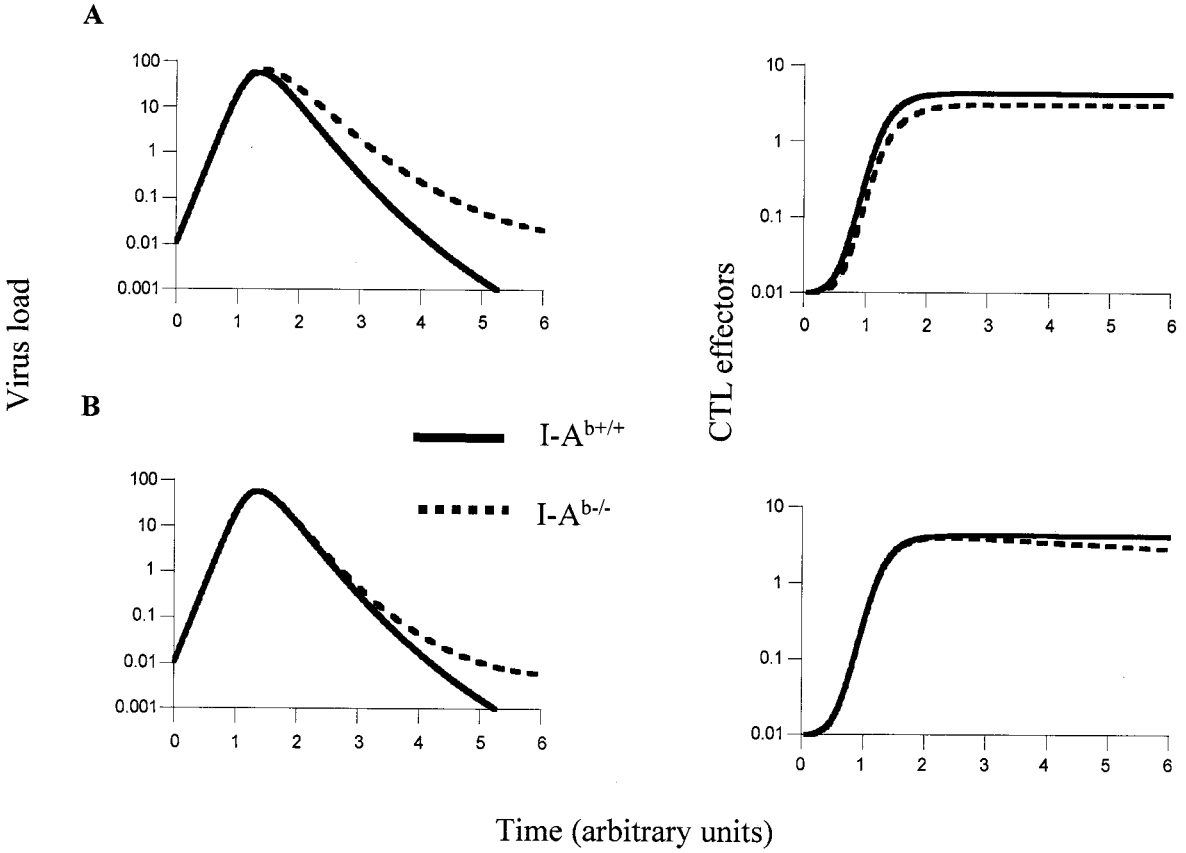

FIG. 5. Simulation of infection in *I-A*$^{b+/+}$ and *I-A*$^{b-/-}$ hosts according to the mathematical model discussed herein. It is assumed that the *I-A*$^{b-/-}$ mice lacking help from CD4$^+$ T cells differ from the *I-A*$^{b+/+}$ controls in two parameters: the rate of expansion (A) and the life span (B) of their CTLs. Both simulations predict similar dynamics. The infection is resolved less efficiently in *I-A*$^{b-/-}$ mice than in *I-A*$^{b+/+}$ mice. At the same time, the CTL dynamics are similar in the presence and absence of T-cell help. The chosen values of parameters were as follows: λ was 10; *d* was 0.1; β was 0.08; *a* was 0.2; and *p* was 1. (A) *c* equals 0.1 in wild-type hosts and 0.05 in mutant hosts; (B) *b* equals 0.01 in wild-type hosts and 0.1 in mutant hosts.

load. If the CTL response is weaker, the infection is resolved more slowly, resulting in higher virus load. The net consequence is that comparable numbers of CTL effectors are generated for each scenario. Similar patterns have been found for human T-cell leukemia virus type 1 infection (28). Thus, consistent with the data, differences in the levels of strength of the CTL response can result in modified viral load kinetics but similar CTL effector responses.

More-complex models taking into account both memory and effector CTLs (40) suggest that differences in the levels of strength of the CTL response can lead to similar effector dynamics but divergent levels of memory generation. This is especially true if the difference lies in the life span of the memory CTLp in the absence of the antigen, which could be a factor influenced by the availability of help. The preceding interpretation is consistent with the data presented here: the levels of effector function following secondary challenge look comparable for the *I-A*$^{b+/+}$ and *I-A*$^{b-/-}$ mice, while memory is significantly reduced in the CD4$^+$-T-cell-deficient animals.

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