

Fig. 2 Correlation of CD8 and $V_{\beta 6}$ expression on cortisone-resistant thymocytes from anti-CD4-treated and control Mls^a congenic Balb/c mice. (Balb/c × Balb.D2.Mls^a)F₁ mice were treated from birth with anti-CD4 (d) or control anti-DNP mAbs (c) and injected with hydrocortisone. Recovered cortisone-resistant thymocytes were double-stained with anti- $V_{\beta 6}$ mAb 44-22-1 and anti-CD8 mAb H35-17.2. Cortisone-resistant thymocytes from untreated Balb/c (a) and (Balb/c × Balb.D2.Mls^a)F₁ (b) mice are included for comparison. CD8⁺ cells accounted for 33.1%, 23.2%, 20.6% and 46.5% of the total in a–d respectively. The proportion of $V_{\beta 6}$ cells in the corresponding subsets was 10.6% (CD8⁺) and 10.7% (CD8[−]) in a, and 4.1% (CD8⁺) and 4.3% (CD8[−]) in d.

Methods. Neonatal injections and cortisone treatment were as in Table 1. Staining with mAb 44-22-1 (see Fig. 1) was followed by biotinylated anti-CD8 mAb H35-17.2 (2 μg) and PE-conjugated avidin (10 μg). Samples were analysed as in Fig. 1.

with the accessory function of CD4 (acting either as an adhesion-strengthening molecule¹³ or as part of the functional TCR complex¹⁴) during the interaction of TCRs on $V_{\beta 6}$ CD4⁺CD8⁺ precursors with Mls^a/class II MHC molecules on thymic non-lymphoid cells. In the most straightforward model, such interference would neutralize the negative selection mechanism and thereby allow further differentiation into $V_{\beta 6}$ CD8⁺ mature T cells. Alternatively, because Mls^a reactivity is strongly correlated with CD4 expression¹⁵, it is possible that a positive selection process in Mls^a mice diverts most $V_{\beta 6}$ CD4⁺CD8⁺ precursors to the CD4 lineage before negative selection. In this situation, anti-CD4 mAb treatment would block the dominant positive selection and again allow $V_{\beta 6}$ CD8⁺ mature T cells to develop.

The nature of the CD8⁺ $V_{\beta 6}$ cells 'rescued' by neonatal anti-CD4 mAb treatment requires further study. Although these cells are likely to be CD4⁺ (CD4⁺ and CD8⁺ cells account for more than 95% of cortisone-resistant thymocytes in normal mice¹⁰), the possibility that they belong to an aberrant (cortisone-resistant) subset of CD4[−] thymocytes cannot be excluded. If indeed CD4[−] $V_{\beta 6}$ cells do arise after anti-CD4 mAb treatment, then clonal deletion can be subverted without concomitant loss of the CD4⁺ T-cell lineage (because putative positive selection mechanisms requiring CD4 are blocked). The mechanism underlying this puzzling dichotomy is unclear, but could be related to differing affinity thresholds for interactions between TCRs and/or CD4 with their corresponding ligands during negative and presumed positive selection events.

We demonstrate that clonal deletion of self-reactive cells (at least in the Mls^a system) can be prevented by neonatal administration of anti-CD4 mAbs. Furthermore, subject to the caveats mentioned above, our results indicate that the primary target of anti-CD4 mAbs in this system is the CD4⁺CD8⁺ thymocyte subset. These results are therefore compatible with the idea that physiological T-cell tolerance occurs at the CD4⁺CD8⁺ stage of development and that CD4⁺CD8⁺ thy-

mocytes contain the precursors of both the CD4⁺ and CD8⁺ mature T-cell lineages.

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Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins

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Several polypeptides are secreted into the medium of cells infected with vaccinia virus, a cytoplasmic DNA virus belonging to the poxvirus family. One of these, a polypeptide of relative molecular mass 19,000¹ is structurally related to epidermal growth factor^{2–4} and binds to epidermal growth factor receptor stimulating proliferation of uninfected cells *in vitro*^{6–7} and *in vivo*⁸. Here, we show that a second, and much more abundant secretory polypeptide, is also encoded by vaccinia virus and is structurally related to the superfamily of complement control proteins. Members of this family can block complement-mediated induction of the inflammatory response, and engulfment, killing and lysis of bacteria and viruses^{9,10}.

Earlier studies¹¹ indicated that two major polypeptides of relative molecular mass (M_r) 35,000 (35K) and 12,000 (12K) are present in the medium of cells infected with vaccinia virus. We confirmed this, and discovered that neither polypeptide was secreted by cells infected with an attenuated mutant of vaccinia virus (designated 6/2)¹² which has a large deletion near the left end of the genome (Fig. 1). These results indicated that both secretory polypeptides were encoded in the region that had been deleted. The 35K protein was purified from the medium of cells infected with wild-type virus and the NH₂-terminal sequence was determined. This was compared with each of the 17 open reading frames, deduced from the nucleotide sequence of the DNA segment deleted in the mutant virus (G. Kotwal, unpublished), and one was found which matched exactly (Fig. 2).

This sequence started downstream of an initiating methionine indicating that during translation a signal peptide of 19 amino acids had been cleaved. The entire open reading frame, of 263 amino acids, has a calculated mass of 28.6K. The apparent over-estimation by polyacrylamide gel electrophoresis, of the

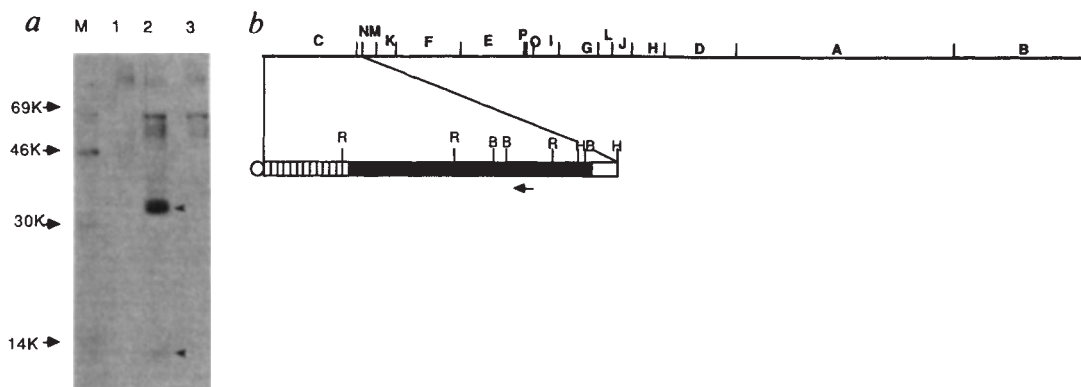
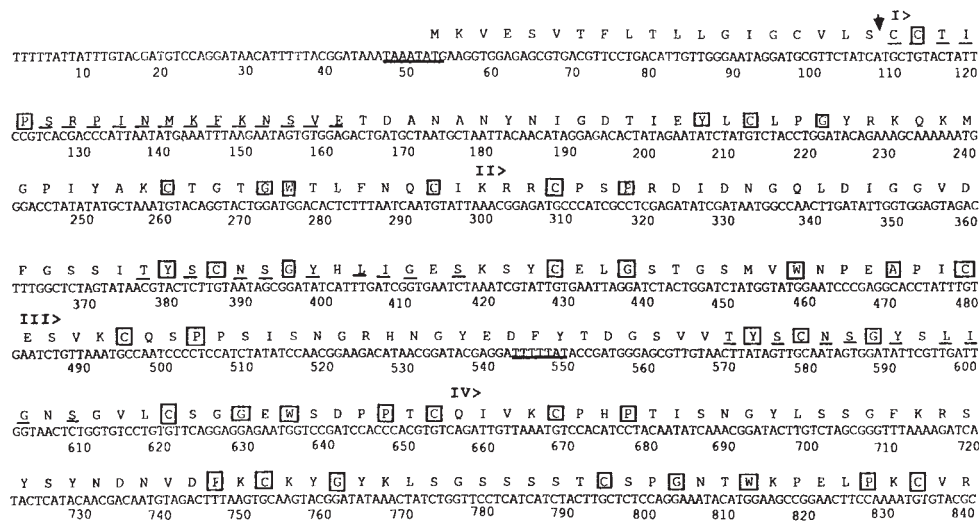


Fig. 1 Proteins secreted by cells infected with wild-type vaccinia virus or deletion mutant 6/2. *a*, An autoradiograph of a polyacrylamide gel is shown. Lanes are: M, ¹⁴C-labelled relative molecular mass marker proteins; medium from rabbit kidney (RK-13) cells that were 1, uninfected; 2, infected with wild-type vaccinia virus; 3, infected with vaccinia virus deletion mutant 6/2. Arrows point to 35K and 12K polypeptides secreted only from cells infected with wild-type vaccinia virus. *b*, Representation of the *Hind*III map¹³ of the genome of vaccinia virus. The left end of the genome is enlarged; the terminal repeat region is indicated by vertical bars; the deleted region of 6/2 is indicated by the shaded area; R, B and H refer to *Eco*RI, *Bam*HI and *Hind*III sites, respectively. The arrow indicates the direction and position of the open reading frame corresponding to the 35K protein.

Methods. RK-13 cells were infected at a multiplicity of infection of 50 with either wild-type vaccinia virus (strain WR) or a deletion mutant of the WR strain (6/2) in minimal medium (RPMI). After 1 h, 50 μCi ml⁻¹ of [³⁵S]methionine was added. The incubation was continued for 15 h, the medium harvested, and the secreted proteins prepared for SDS-polyacrylamide gel electrophoresis.

Fig. 2 Nucleotide sequence of the DNA including and flanking the gene encoding the 35K protein. The order of amino acids, represented in the one letter code, was deduced from the nucleotide sequence. The site of cleavage of the signal peptide (indicated by a downward arrow) was determined from the NH₂-terminal sequence of the secreted protein. The underlined amino acids beginning with T indicate the location of a repeated sequence. The amino acids that are boxed conform to the consensus sequence of the 60-amino-acid repeat superfamily which is shown at the bottom of the figure. Roman numerals I, II, III and IV indicate the start of the four-tandem 60-amino-acid repeating units. Nucleotide sequences similar to a late transcription start site (TAAATATG)¹⁴ and an early transcription termination signal (T₃NT)¹⁵, that frequently occurs in the middle of late genes, are underlined.



CONSENSUS SEQUENCE OF 60-AMINO-ACID REPEAT SUPERFAMILY:
 ---C--P-----Y/F-C--G-----C--G-W---A/P-C-
 1 60

Methods. DNA fragments were cloned in M13 vectors, digested with exonuclease III to generate overlapping segments, and the nucleotide sequence of both DNA strands was determined by the dideoxy chain termination method. The secreted 35K protein was separated on a 12.5% SDS-polyacrylamide gel and transferred to an Immobilon filter. The NH₂-terminal sequence was determined with an Applied Biosystems 477A gas-phase protein sequencing system. The result of the first 18 cycles was -, -, T, I, P, S, R, P, I, N, M, K, N, S, V, E. Because cysteines are not detectable by this method, their release in the first two cycles could only be inferred.

size of the processed polypeptide is not due to glycosylation because there are no glycosylation sites; moreover incorporation of glucosamine was not detected (G. Kotwal, unpublished). The deduced amino acid sequence of the secretory polypeptide revealed internal repetitions of which the most striking was Thr-Tyr-Ser-Cys-Asn-Ser-Gly-Tyr-His/Ser-Leu-Ile-Gly-Glu/Asn-Ser. This occurred between amino acids 90-103 and 154-167, numbered from the NH₂ terminus of the mature polypeptide (Fig. 2). In addition, conserved elements of the 60-amino-acid repeating unit of a superfamily of structurally related eukaryotic proteins^{9,10} are present in four places, comprising the entire secreted form of the vaccinia polypeptide (Fig. 2). This superfamily contains at least 6 complement control pro-

teins, of which 5 interact with C3b or C4b, and three non-complement proteins.

A search of the NBRF protein data base and GenBank revealed that the protein with the greatest similarity to the vaccinia virus 35K secretory protein is the human C4b-binding protein which contains eight 60-amino-acid repeating units¹⁶. The smaller vaccinia protein has a 38% identity with the first half of C4b (Fig. 3) and a 28% identity with the second half (data not shown). Moreover, in the homologous regions, 16 out of the 17 cysteines are conserved, as are the majority of prolines and glycines, indicating that the structural similarities may be even greater than the sequence ones (Fig. 3). Interestingly, the only gap of more than one amino acid which was inserted into

