Immunology 1995 **84** 153–158

Characterization of defectiveness in endogenous antigen presentation of novel murine cells established from methylcholanthrene-induced fibrosarcomas

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SUMMARY

Three cell lines (4A1, 4C2 and 6D1 cells) derived from fibrosarcoma induced by the inoculation of 3-methylcholanthrene into C3H/HeN (H-2^k) mice were examined for their ability to present antigens to $CD8^+$ cytotoxic T lymphocytes (CTL). 6D1 and 4C2 cells were deficient in presenting endogenously synthesized influenza virus antigens to CTL, but they were able to present antigens when they were sensitized with a synthetic epitope peptide. The expression of the H-2 K^k gene in 4C2 and 6D1 cells was much reduced and was detectable only with Northern blot hybridization. The expression of two transporter genes (*TAP1* and *TAP2*), examined by Northern hybridization, was also reduced in both cells, and negligible particularly in 4C2 cells. Interferon-γ (IFN-γ) treatment of these cells induced expression of K^k, *TAP1* and *TAP2* genes and rescued the defect of class I-restricted antigen presentation in 4C2 and 6D1 cells. Even after this treatment, however, antigen-presentation capability of 4C2 cells was still much lower than that of normal 4A1 cells. This finding suggests that 4C2 cells might have an additional defective gene(s), whose products are involved in the processing of class I-restricted antigen, besides the K^k and *TAP* genes, and this may explain the difficulty of 4C2 cells to induce tumour-specific immunity, as described previously. To our knowledge, the 4C2 cell is the first tumour cell postulated to have more than three defective genes involved in class I-restricted antigen presentation.

INTRODUCTION

$CD8^+$ cytotoxic T lymphocytes (CTL) are an essential element of the immune response against the infection of intracellular pathogens such as viruses, and against tumours. They recognize a heterotrimeric complex, consisting of a major histocompatibility complex (MHC) class I heavy chain, a light chain (β_2-microglobulin) and an antigenic peptide with the length of 8–10 residues.[1,2] The antigenic peptides or their precursors are produced in the cytoplasm by the cleavage of endogenously synthesized proteins, which might be conducted by the proteasome.[3–5] Peptides are then translocated into the lumen of the endoplasmic reticulum (ER) by the transporter consisting of the heterodimer of *TAP1* and *TAP2* (transporter associated with antigen processing; TAP).[6,7] In the lumen of ER, the peptides assemble with class I heavy and light chains to make a ternary complex, and the complex is transported to the cell surface.[8–10]

Class I molecules are poorly expressed on the surfaces of a number of tumour cells. This gives tumour cells a reduced antigen presentation capability to $CD8^+$ CTL, and it is believed that this is a mechanism for tumours to escape immune surveillance.[11–13] Although in most of these tumour cells transcription of the class I gene is down-regulated, it has been shown recently that the suppression of *TAP1* and *TAP2* gene expression is another strategy for tumour cells to reduce the expression of class I molecules on their surfaces and reduce antigen-presentation capability.[14,15] Involvement of some gene(s) other than *TAP1* and *TAP2* in the defective antigen presentation has not been excluded.

Many cell lines have been established from 3-methylcholanthrene-induced fibrosarcomas in C3H/HeN mice (H-2^k).[16] In the present study, using three such cell lines that are different in the expression of class I molecules on their cell surface, we examined their capabilities to present endogenous virus antigens, and analysed the causes of the defect in antigen presentation in two of them.

Received 10 June 1994; revised 15 August 1994; accepted 2 September 1994.

Abbreviations: CTL, cytotoxic T lymphocyte; NP, nucleoprotein; TAP, transporter associated with antigen processing.

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MATERIALS AND METHODS

Virus and cells

Influenza virus strain A/PR/8/34 (PR8), grown in the allantoic cavity of embryonated eggs, was used.

4A1, 4C2 and 6D1 cells have been described previously.[16] They were maintained in RPMI-1640 medium with 10% fetal calf serum (FCS). The three cell lines were chosen from many lines in terms of MHC class I expression and induction of protective immunity on *in vivo* inoculation. 4A1 cells expressed a high level of class I antigens and induced anti-tumour protective immunity. 4C2 and 6D1 cells expressed only a slight amount of class I molecules, and only 6D1 cells induced protective immunity.[16]

L929 cells derived from C3H mice were maintained in minimal essential medium (MEM) with 10% FCS.

CTL and CTL clones

PR8 virus nucleoprotein (NP)-specific monoclonal CTL, NP102, was established by a method described previously[17] and used throughout the present experiment.

Cytotoxicity assay

Target cells were loaded with $Na_2{}^{51}CrO_4$ overnight and then infected with influenza virus for 4–5 hr or incubated with NP50-57 (SDYEGRLI), which is the K^k-restricted T-cell epitope in NP of PR8 (our unpublished data),[18,19] for 30–120 min at 37°. Sensitized target cells were mixed with CTL, and cell lysis was determined by a 4-hr ^{51}Cr-release assay. Percentage specific lysis was calculated as follows: 100 × [(experimental c.p.m. − spontaneous c.p.m.)/(maximal c.p.m. − spontaneous c.p.m.)].

Metabolic labelling and immunoprecipitation

Cells were incubated at 37° for 30 min in methionine- and cysteine-free MEM and then labelled with EXPRE^{35}S^{35}S (Du Pont NEN Products, Boston, MA) (3·7 MBq/ml) for 1–2 hr. Labelled cells were lysed with RIPA-buffer (0·5% Nonidet P40, 150 mM NaCl, 50 mM Tris–HCl, pH 7·5, 0·5 U/ml aprotinin) for 30 min on ice and centrifuged at 45 000 *g* for 30 min. After non-specifically binding materials to protein A had been removed, the supernatant was incubated with anti-K^k monoclonal antibody (mAb) 370, which was kindly supplied by Dr T. Sakuma (Institute for Molecular Biology, Meiji Nyugyo Co. Ltd), for 2 hr at 4°, and then protein A–sepharose was added and further incubated for 1 hr at 4°. The immune complex was washed with RIPA-buffer four times and then analysed by SDS–PAGE using 13% polyacrylamide gel. Protein bands were visualized by fluorography using EN^{3}HANCE (Du Pont NEN Products) and Kodak XAR-5 X-ray film (Eastman Kodak, Rochester, NY).

Probes for Northern blot analysis

To obtain specific probes, cDNA of TAP1 and TAP2 mRNA was cloned. First-strand cDNA was synthesized from poly(A)$^+$ RNA isolated from L929 cells by using TAP1- and TAP2-specific primers that had the sequence AGTCCAGAGGCCTTGTCAGT and TGCTCTTCCCTG-ACCCATTG, respectively. The TAP1- and TAP2-specific DNA fragments were then amplified by polymerase chain reaction (PCR) with the primers that had the sequence TGGAGACATGCTGTGTCGGA for TAP1 and TCAGAA-GCTCTCCTGAGGTG for TAP2. The PCR products were then cloned into the *Sma*I site of pBluescript SK(−) (Stratagene La Jolla, CA). The identity of the cloned DNA was confirmed by nucleotide sequencing with a DNA-sequencer (SQ-3000; Hitachi Electronics Engineering Co., Tokyo, Japan). The cloned cDNA of β-actin was kindly provided by Dr K. Tokunaga. The ^{32}P-labelled probes were prepared from the cloned cDNA by the random priming method.

The detection of K^k mRNA was done with ^{32}P-labelled K^k-specific synthetic oligonucleotide (GGCGATCTGCGTGT-TCCGCTCCC), as used by D. Klar and G. J. Hämmerling.[20]

Northern blot hybridization

Poly(A)$^+$ RNA was isolated using Quick Prep Micro (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's manual. For Northern blot, 2–5 μg of poly(A)$^+$ RNA was subjected to electrophoresis in 1·5% agarose formaldehyde gel and transferred to a nylon membrane (BIODYNE B; Pall, East Hills, NY) by the vacuum blotting method using VacuGene XL (Pharmacia Biotech). After hybridization with a labelled probe, the membrane was washed under high stringency conditions, and subjected to autoradiography.

RESULTS

Presentation of endogenous antigen

4A1, 4C2 and 6D1 cells were infected with influenza virus and tested for lysis by the NP-specific K^k-restricted CTL clone NP102. While the virus-infected 4A1 cells were efficiently lysed, 6D1 cells were only slightly lysed and 4C2 cells were negligibly lysed: 73% lysis in 4A1 cells and 18% in 6D1 cells at an effector : target (E : T) ratio of 20 : 1 (Fig. 1).

The immunoprecipitation with anti-PR8 serum from the lysates of pulse-labelled virus-infected cells showed similar amounts of synthesis of viral proteins in 4A1, 4C2 and 6D1 cells (data not shown). Therefore, the defects in antigen presentation in 4C2 and 6D1 cells were not due to the poor synthesis of viral proteins.

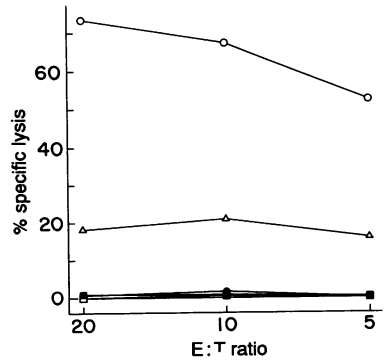

Figure 1. Recognition of influenza virus-infected tumour cells by a NP-specific CTL clone. ^{51}Cr-loaded 4A1 (○), 6D1 (△) and 4C2 (□) cells were infected with PR8 virus at 37° for 4 hr. Cytolysis of target cells by the NP-specific CTL clone, NP102, was determined by 4-hr ^{51}Cr-release assay. Cytolysis of uninfected 4A1 (●), 6D1 (▲) and 4C2 (■) cells was also determined.

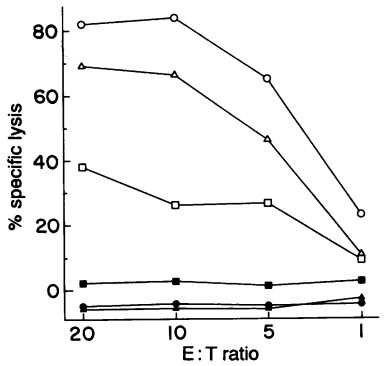

Figure 2. Cytolysis of peptide-pulsed tumour cells by a NP-specific CTL clone. 4A1 (○, ●), 6D1 (△, ▲) and 4C2 (□, ■) cells were incubated with $Na_2{}^{51}CrO_4$ in the presence of 100 μM NP50-57 peptide (○, △, □) or in the absence of peptide (●, ▲, ■) at 37° overnight. Cytolysis of ^{51}Cr-loaded cells by NP102 was measured by a 4-hr ^{51}Cr-release assay.

Recognition of exogenous antigenic peptide by CTL

We also examined whether 6D1 and 4C2 cells were able to present antigens to CTL when an epitope peptide was provided exogenously. 4A1, 4C2 and 6D1 cells were incubated overnight in the presence of 100 μM of NP50-57 peptide, which is a K^k-restricted epitope of PR8 NP protein, and then cytotoxicity of NP102 to these peptide-pulsed cells was assayed (Fig. 2). The CTL lysed not only 4A1 cells but also 4C2 and 6D1 cells. The extent of lysis, however, was different depending on the cells. At an E:T ratio of 20:1, 82%, 69% and 38% lysis was observed for 4A1, 6D1 and 4C2 cells, respectively.

Synthesis of K^k molecules

To elucidate the cause of differences in antigen-presentation efficiency among 4A1, 4C2 and 6D1 cells, the synthesis of K^k molecules in these cells was compared. Figure 3 shows the patterns of immunoprecipitation with anti-K^k mAb 370 from the detergent extracts of 4A1, 6D1 and 4C2 cells. Whereas K^k molecules were precipitated from 4A1 cells in the form of a complex with the light chain (β_2-microglobulin), no K^k was precipitated from 4C2 and 6D1 cells. The absence of K^k molecules was not due to the specificity of the mAb, because none of the six different anti-K^k mAb we tried precipitated K^k molecules from 4C2 and 6D1 cell lysates. Furthermore, the addition of the epitope peptide (NP50-57) and β_2-microglobulin to the cell lysates did not enhance the precipitation to a detectable level (data not shown). This procedure induces a change in the antigenicity caused by the heterotrimer formation of class I molecules in TAP-deficient cells such as the RMA/S cell.[21–24]

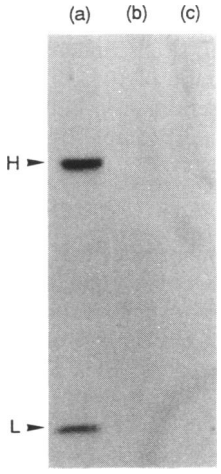

Figure 3. Synthesis of K^k molecules in tumour cells. K^k molecules were immunoprecipitated with anti-K^k mAb 370 from the cell lysates of 4A1 (a), 6D1 (b) and 4C2 (c) cells pulse-labelled with ^{35}S-Met/Cys at 37° for 2 hr. The immunoprecipitates were analysed on SDS–PAGE using a 13% polyacrylamide gel. The gel was processed for fluorography. H-2 K^k class I heavy chain (H) and light chain of β_2-microglobulin (L) are indicated.

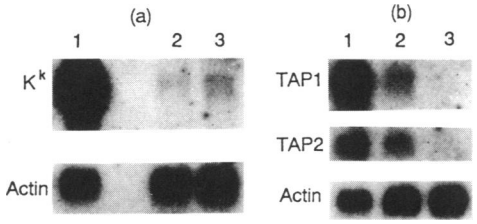

Figure 4. Northern blot analysis of tumour cell mRNA with four different probes. Five micrograms of poly(A)$^+$ RNA isolated from 4A1 (1), 6D1 (2) and 4C2 (3) cells was separated on a 1·5% agarose formaldehyde gel and transferred to a nylon membrane. A pair of membranes was prepared; one membrane was hybridized with a K^k-specific synthetic oligonucleotide probe (a) and the other one was hybridized with a TAP1-specific probe and then rehybridized with a TAP2-specific probe (b). Both membranes were finally rehybridized with an actin-specific probe to control the amount of RNA loaded.

Although no K^k molecules were detected in 6D1 and 4C2 cell lysates, at least a minor dose of K^k molecules should have been expressed on the surfaces of 4C2 and 6D1 cells, because these cells did present the exogenous antigenic peptide to K^k-restricted CTL (Fig. 2). Probably, the minor dose of empty class I molecules, whose amount was insufficient to be detected by the immunoprecipitation method, was enough to make the cells susceptible to CTL-mediated cell lysis. To examine this possibility, the expression level of the K^k gene was assessed by Northern blot analysis of poly(A)$^+$ RNA isolated from 4A1, 4C2, and 6D1 cells. Hybridization with a K^k probe showed that mRNA of the K^k gene did exist in 4C2 and 6D1 cells, although the amount was very small compared to 4A1 cells (Fig. 4a).

Northern blot analysis of *TAP1* and *TAP2* genes

Since most of the cells capable of presenting only extracellularly provided peptides have been reported to be deficient in *TAP* genes or their expression,[9,10] we investigated whether 6D1 and 4C2 cells had a similar defect besides reduced K^k gene expression.

Northern blot analysis using cDNA probes of *TAP1* and *TAP2* genes showed that the expression of *TAP1* and *TAP2* genes was reduced in 4C2 and 6D1 cells compared with 4A1 cells (Fig. 4b). We did not detect any mRNA of *TAP1* and *TAP2* genes in 4C2 cells, while we did detect minor doses in 6D1 cells. This low expression of *TAP* genes might also contribute to the deficit of 4C2 and 6D1 cells in antigen presentation.

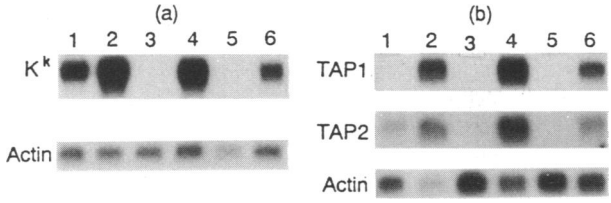

Figure 5. Northern blot analysis of IFN-γ-treated tumour cell mRNA with four different probes. 4A1 (1, 2), 6D1 (3, 4) and 4C2 (5, 6) cells were incubated with 0·1 μg/ml of recombinant mouse IFN-γ for 48 hr (2, 4, 6) at 37° or incubated without IFN-γ (1, 3, 5). Three micrograms of poly(A)$^+$ RNA isolated from the treated cells was processed as described in Fig. 5. A pair of membranes was prepared and they were also similarly hybridized with a K^k-specific synthetic oligonucleotide probe (a), and TAP1- and TAP2-specific probes (b). They were finally rehybridized with an actin-specific probe.

Interferon-γ treatment induces expression of *K^k*, *TAP1* and *TAP2* genes

Interferon-γ (IFN-γ) treatment induces or enhances expression of various genes residing in MHC class I and class II loci, including all three genes *K^k*, *TAP1* and *TAP2*.[25–27] If the defective presentation of endogenous antigen in 4C2 and 6D1 cells is caused by low-level expression of the three genes, IFN-γ treatment is expected to rescue this defect.

Northern blot hybridization confirmed that the expression of *K^k*, *TAP1* and *TAP2* genes was increased in IFN-γ treated 4A1, 4C2 and 6D1 cells (Fig. 5). The expression of the three genes in treated 6D1 cells was comparable to that of treated 4A1 cells. That of treated 4C2 cells was less than that of treated 4A1 cells, but comparable or more than untreated 4A1 cells.

Translation of IFN-γ induced K^k mRNA was examined by immunoprecipitation with anti-K^k mAb. In the treated 4C2 and 6D1 cells, the complex of K^k molecules and β_2-microglobulin was detected (Fig. 6). The amount of precipitated K^k molecules in 6D1 cells was less than that in treated 4A1 cells and comparable to that in untreated 4A1 cells, although a comparable amount of K^k mRNA was detected in IFN-γ-treated 4A1 and 6D1 cells (Fig. 5).

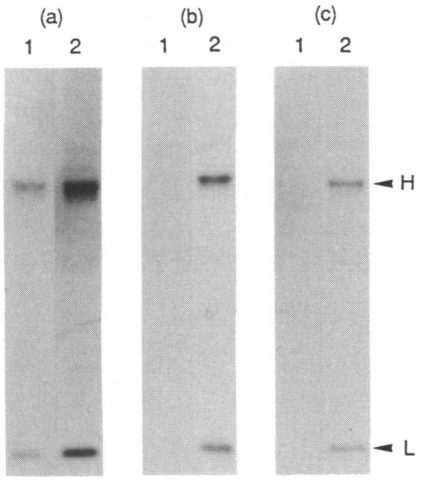

Figure 6. Induction of K^k molecule synthesis in IFN-γ-treated tumour cells. 4A1 (a), 6D1 (b) and 4C2 (c) cells were treated with IFN-γ (2) or untreated (1) and labelled with ^{35}S-Met/Cys. Immunoprecipitates by anti-K^k mAb 370 were similarly processed as described in Fig. 3. Indications H and L are the same as in Fig. 3.

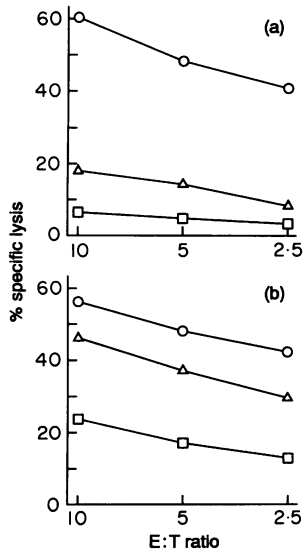

Figure 7. Recovery of endogenous antigen presentation in IFN-γ-treated tumour cells. 4A1 (○), 6D1 (△) and 4C2 (□) cells were IFN-γ treated (b) or untreated (a) and then loaded with ^{51}Cr. Loaded cells were infected with PR8 virus for 4 hr and tested for their cytolysis by NP102. Cytolysis was measured by 4 hr-^{51}Cr-release assay.

IFN-γ treatment rescues the defect of antigen presentation

The effect of IFN-γ treatment on endogenous antigen presentation was also investigated. Treated 4A1, 4C2 and 6D1 cells were infected with influenza virus, and cytolysis by NP102 was determined. Treated 6D1 cells were lysed with comparable efficiency to 4A1 cells (Fig. 7). Treated 4C2 cells were also lysed by the CTL, although the efficiency was still less than that of other cells: about 60% lysis of 4A1 cells, about 50% lysis of treated 6D1 cells, and about 20% lysis of treated 4C2 cells at an E:T ratio of 10:1. These data show that the defect of antigen presentation in 6D1 cells but not 4C2 cells could be overcome almost completely by IFN-γ treatment.

DISCUSSION

The present study demonstrates that the novel cell lines 6D1 and 4C2, which express only marginal amounts of MHC class I molecules, were deficient in endogenous antigen presentation to class I-restricted CTL, but capable of presenting exogenously provided antigenic peptides. This finding is reminiscent of *TAP*-gene deficient cells such as RMA/S and T2 cells, which express a reduced level of class I molecules on their surfaces and are able to present only exogenous antigenic peptides.[9,10] The expression of *TAP* genes was also impaired in 4C2 and 6D1 cells. Although 4C2 and 6D1 cells have phenotypes similar to those of TAP-deficient cells, there are some differences. Whereas RMA/S cells present exogenous antigens with almost the same efficiency as wild-type parent cells,[23] 4C2 and 6D1 cells presented exogenous antigens less efficiently than 4A1 cells, which expressed normal amounts of class I molecules

(Fig. 2; our unpublished data). We presumed that the low-level transcription of the K^k gene might be the main cause of the inefficient presentation of exogenous antigenic peptide in 4C2 and 6D1 cells.

Moreover, there were some differences between the two cell lines 6D1 and 4C2. Northern blot hybridization analysis revealed that the transcriptions of *TAP1* and *TAP2* genes were not detectable in 4C2 cells, but a significant amount was detected in 6D1 cells. IFN-γ treatment enhanced the expression of the three genes K^k, *TAP1*, *TAP2*, caused formation of a complex of class I heavy chain and β_2-microglobulin, and further recovered endogenous antigen presentation in the two cell lines. However, recovery of treated 4C2 cells was only partial, while that of treated 6D1 cells was almost similar to the level of untreated 4A1 cells. We assumed that 4C2 cells might have an additional defective gene(s), or defective expression of an additional gene(s), whose products are involved in class I-restricted antigen presentation. Possible candidates of such a gene are *LMP2* and *LMP7* genes, whose products are suspected to be involved in antigenic peptide production, although cells defective in both genes still present antigen to $CD8^+$ CTL.[29,30] We hope further analysis of 4C2 cells will cast some light on understanding the class I-restricted antigen presentation mechanism in more detail. We also believe that the defective presentation of 6D1 cells was mainly or exclusively due to the low level of expression of the K^k gene. Our preliminary experiments on the introduction of the K^k gene into 6D1 but not 4C2 cells consistently showed an almost complete recovery of the antigen-presentation capability.

As previously reported,[16] 4C2 cells did not induce anti-tumour protective immunity, while 6D1 cells did in spite of a marginal expression of a K^k gene at a level similar to that in 4C2 cells. This may be explained by the reduced expression of *TAP* genes in 4C2 cells; 6D1 cells might have class I molecules with antigenic peptides on the cell surface, whereas 4C2 cells might have class I molecules without peptides on the surface. Thus, putative tumour resistance antigen–peptide associated with class I molecules on the surface of 6D1 cells might be enough to induce tumour-specific protective immunity. The reduced antigen presentation capability in tumour cells such as 4C2 and 6D1 should be considered for the understanding the tumourgenesis, because it might be closely related to tumour evasion of host defence mechanisms.

ACKNOWLEDGMENTS

We thank Dr Hiroshi Minakata (Suntory Institute for Bioorganic Research) and Dr Kozabro Sato (Shionogi Research Institute) for kindly providing the synthetic peptide NP50-57 and recombinant IFN-γ, respectively.

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