Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 8723–8727, November 1986
Immunology

Expression of major histocompatibility complex class I antigens as a strategy for the potentiation of immune recognition of tumor cells

(immunosurveillance/tumor rejection)

KENICHI TANAKA*, HIROAKI HAYASHI*, CHUYA HAMADA†, GEORGE KHOURY*, AND GILBERT JAY*

*Laboratory of Molecular Virology, National Cancer Institute, Bethesda, MD 20892; and †Department of Virology, Niigata University School of Medicine, Niigata, Japan

Communicated by Martin Gellert, August 5, 1986

ABSTRACT **Like many primary tumors, human adenovirus type 12 (Ad12)-transformed mouse cells express greatly reduced levels of the major histocompatibility complex (MHC) class I antigens and are highly tumorigenic in immunocompetent hosts. Expression of a transfected class I gene by these cells can abrogate their tumorigenicity. Both the *K* and the *L* class I genes can suppress the malignant phenotype. Previous studies showed that interferon can induce class I gene expression in certain Ad12-transformed cells and can suppress their tumorigenic phenotype. We now demonstrate that preimmunization of mice with a nontumorigenic dose of interferon-treated Ad12-transformed tumor cells can afford protection against a subsequent challenge by a tumorigenic dose of untreated Ad12-transformed tumor cells. Similar immunity can also be induced by using cells transfected with the *K* gene, and the observed protection appears specific to Ad12-transformed cells. Significant protection can be achieved even if immunization is provided subsequent to the tumor challenge. Since increasing numbers of human tumors have been found to have reduced levels of MHC class I antigens, the prospect of therapy by immunization with the parental tumor cells that have been manipulated to induce class I gene expression offers an attractive experimental model.**

Transformation of cells by human adenovirus type 12 (Ad12) is a valuable system for the study of tumorigenesis (1, 2). Unlike most cells transformed in culture, those transformed by Ad12 are highly tumorigenic in immunocompetent recipients. Like many primary tumors, Ad12 transformants express greatly reduced or undetectable levels of the major histocompatibility complex (MHC) class I antigens.

An established role of the MHC class I molecules is to present tumor cells bearing foreign antigens to the immune system (3). The suppression of class I gene expression upon Ad12 transformation is thought to explain the high tumorigenic phenotype of these cells. This assumption was confirmed by the finding that expression of a cloned class I gene introduced by DNA-mediated gene transfer can completely abrogate the tumorigenicity of Ad12-transformed cells (4). Thus, suppression of class I gene expression is a requisite for the induction of malignancy by Ad12.

Since the class I antigens are encoded by a multigenic family (5, 6), it seemed valuable to define whether differences exist among them with regard to their ability to reverse Ad12 tumorigenicity. More importantly, we wished to determine whether tumor cells transfected with a class I gene can immunize animals against the parental Ad12-induced tumor not expressing any class I antigen. The present study addresses these two questions.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Mice. C57BL/6 and (BALB/c × C57BL/6)F_1 mice were obtained from The Jackson Laboratory.

Cell Cultures. All cells were maintained at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The α and β interferon preparations (IFN-α and IFN-β, 1.3 × 10^7 IRU/mg) were obtained from Lee BioMolecular Research Laboratories (San Diego, CA) (7).

DNA Transfection. Ten micrograms of pI255 [which contains the entire K^b gene inserted at the *Sal* I site of pTCF (8, 9)] and 0.1 μg of pRSVneo [which contains the gene encoding neomycin resistance under the control of the Rous sarcoma virus promoter (4)] were introduced into C57AT1 cells by the calcium phosphate technique (10). The calcium phosphate coprecipitates were formed by adding a 250 mM $CaCl_2$ solution containing plasmid DNA dropwise to an equal volume of 2× HBS buffer (1× HBS = 25 mM Hepes/0.75 mM Na_2HPO_4/140 mM NaCl, adjusted to pH 7.1) under a stream of N_2. The calcium phosphate DNA precipitates were then added to the cells. Following incubation for about 4 hr, the cells were washed two or three times and left in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Approximately 48 hr after transfection, cells were split into a selective medium containing 0.5 mg of G418 per ml. Ten days later, individual clones were picked and expanded.

RNA Blot Analysis. Poly(A)$^+$ RNA was isolated as described (11). Aliquots of RNA were adjusted to contain 50% formamide, 20 mM 4-morpholinepropanesulfonic acid (Mops, pH 7.0), 5 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde, were heated at 60°C for 10 min, and were subjected to electrophoresis on a 1.0% agarose gel containing 2.2 M formaldehyde. The running buffer was 20 mM Mops, pH 7.0/5 mM sodium acetate/1 mM EDTA, and electrophoresis was performed at 35–40 mA for 4 hr at 4°C. The RNA was transferred to a nitrocellulose filter as described (11). The conditions for hybridization have been described (12).

Detection of Cell Surface Antigens. For the microfluorometry assay, single-cell suspensions obtained from monolayer cultures by treatment with trypsin and EDTA were incubated with a mouse monoclonal antibody specific to the K^b class I antigen and stained with fluorescein-conjugated rabbit anti-mouse IgG (4). The stained cells were analyzed in the Becton–Dickinson FACS analyzer.

Tumor Induction. Mice, in groups of 10, were each given a single subcutaneous injection on the right thigh of a fixed dose of the control or of the appropriately-treated cells. The progressive increase in the size of the tumor mass, assessed by measuring the diameter in centimeters, was determined at regular intervals for each animal. Deaths of individual mice resulting from tumor growths were recorded (4).

Abbreviations: MHC, major histocompatibility complex; Ad12, adenovirus type 12; IFN, interferon.

RESULTS

Transfection of the cloned K^b gene into C57AT1 cells, an established Ad12-transformed C57BL/6 (H-2^b haplotype) cell line that does not express a detectable amount of class I antigens, has led to its expression even in the absence of a heterologous transcriptional enhancer and promoter (Fig. 1). This confirms our previous finding with the L^d gene that an exogenous class I gene can be expressed in Ad12-transformed cells despite the suppression of the endogenous class I genes (4). While the transfected K^b gene is transcriptionally active, individual clones (designated K25, K73, and K74) express various levels of this exogenous gene (Fig. 1*A*, lanes 3–5). Although all of the 20 transfectants tested were positive for the expression of the K^b transcript as compared to the parental C57AT1 cell line (lane 2), none expressed it at a level more than one-third that observed with a BALB/c 3T3 cell line (lane 1). Since the expression of the Ad12 *E1A* gene was not perturbed by the transfection of the K^b gene (Fig. 1*B*), the observed variability and the relatively low level of expression may suggest that the Ad12 function also interferes but not completely with the expression of the exogenous class I gene.

While the parental C57AT1 cells and the G418-resistant C57AT1neo cells are highly tumorigenic in syngeneic C57BL/6 mice, neither K25, K73, nor K74 (three individual clones that express the transfected K^b gene) is capable of inducing tumors, even at a dose of 1×10^6 cells (Fig. 2 *A* and *B*). This observation confirms our earlier finding with the L^d gene that the expression of a transfected class I antigen can reverse the tumorigenic phenotype of Ad12-transformed cells (4).

In order to determine whether differences exist between the *K* and the *L* genes, the tumorigenic potential of two K^b transfectants (K73 and K74) and two L^d transfectants (L99 and R9) were compared (Fig. 2 *C* and *D*). Since the K^b gene was derived from C57BL/6 mice (H-2^b haplotype) and the L^d gene was derived from BALB/c mice (H-2^d haplotype), we were restricted to using the (BALB/c × C57BL/6)F_1 mice for this study. Comparison of the survival curves showed no detectable difference when the parental C57AT1 cells were inoculated into either C57BL/6 mice or (BALB/c × C57BL/6)F_1 mice (cf. Fig. 2 *A* and *C*), thus suggesting that the F_1 hybrids are equally susceptible as recipients of tumor cells. At the appropriate low-cell dose, neither K^b- nor L^d-transfected clones were tumorigenic. When tested at either 3×10^6 cells (Fig. 2*C*) or 1×10^6 cells (Fig. 2*D*), the K^b-transfected clones (K73 and K74) were detectably less tumorigenic than the L^d-transfected clones (L99 and R9). The particular K^b- and L^d-expressing clones were selected for this comparison because they express similar levels of cell-surface class I antigens. Barring differential sensitivity of F_1 mice to different alleles of class I antigens (14), our study suggests that the K antigen appears more efficient than the L antigen in the presentation of the Ad12-transformed C57AT1 cells.

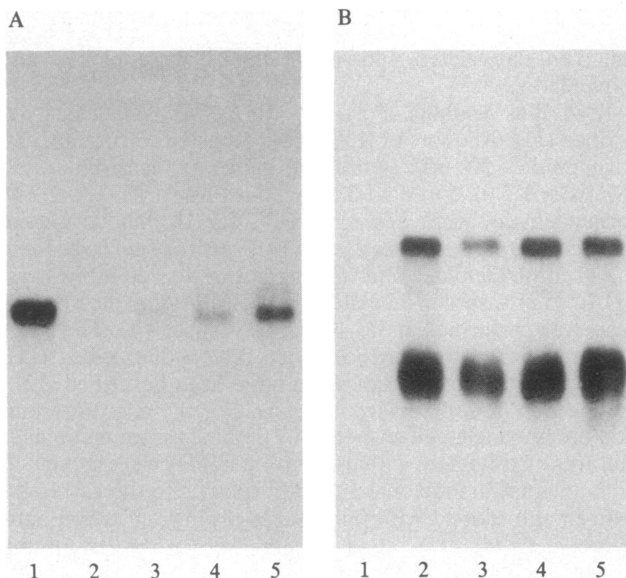

FIG. 1. Characterization by blot-hybridization analysis of mRNA expressed in Ad12-transformed cells transfected with the K^b gene. Equivalent amounts of poly(A)$^+$ RNA obtained from BALB/c 3T3 (lanes 1), C57AT1 (lanes 2), K25 (lanes 3), K73 (lane 4), and K74 (lanes 5) cells were fractionated in a 1.0% agarose gel in the presence of formaldehyde as described (11). The RNA was then transferred from the gel to a nitrocellulose membrane, and the resulting RNA blot was hybridized successively to two ^{32}P-labeled DNA probes (4): an end-labeled oligonucleotide probe that is specific to the *K* gene (12) (*A*), or a nick-translation probe derived from a genomic clone of the Ad12 *E1A* gene (13) (*B*).

Since a majority of the Ad12-transformed cells show a marked suppression but not total repression of MHC class I gene expression (1, 4), as has been observed for certain primary malignancies, it is conceivable that the presence of a low level of class I antigen will suffice for presentation of the tumor cells, provided the immune system can be potentiated toward a more effective recognition of these cells. Although these low levels of class I antigens are not sufficient to allow tumor cells themselves to induce immunity, one possibility would be to immunize animals against a "class I negative" tumor by using isogenic tumor cells that have been rendered class I positive (15–17). By potentiating the immune system to recognize and reject tumor cells bearing "normal" levels of class I antigens, even tumor cells with reduced or subdetectable levels of class I antigens may be destroyed.

To test this model, we elected to use an Ad12-transformed cell-line (C3ATI) that expresses a low residual level of class I antigens but is highly inducible for class I gene expression by treatment with murine IFN (7). While a single dose of 2×10^3 IFN-treated C3AT1 cells (class I positive), injected 7 days in advance, afforded complete protection against challenge by an otherwise tumorigenic dose of C3AT1 cells, a similar dose of untreated C3AT1 cells (class I negative) showed only marginal effect (Fig. 3*A*). This differential activity between the IFN-treated and untreated cells to provide immunity upon a subsequent challenge is even more pronounced when the number of immunizing cells was reduced to 2×10^2 (Fig. 3*B*).

As a further demonstration that immunity against tumor cells can be experimentally induced, we have used the C57AT1 clone, which does not express any detectable endogenous class I antigen, and their isogenic derivatives, which express a transfected K^b gene (see Fig. 1). In addition, we asked whether immunity could be achieved at or subsequent to the tumor challenge.

C57BL/6 mice were each given a tumorigenic dose of 8×10^5 C57AT1 cells (class I negative) at the right thigh and either concomitantly (Fig. 4 *A* and *C*) or 2 days later (Fig. 4 *B* and *D*) were injected at the left thigh with a single nontumorigenic dose of 5×10^5 K^b-transfected C57AT1 cells (class I positive). When the immunization was provided at the same time as the tumor challenge, both K^b-transfected C57AT1 clones (K73 and K74) were able to protect up to 70% of the mice against the parental C57AT1 tumor (Fig. 4*A*). Upon comparison of the average tumor size between the various groups of mice, a biphasic response curve reflecting tumor regression among individuals within the immunized group was observed (Fig. 4*C*). When the immunization was provided 2 days after tumor challenge, the results, although somewhat less impressive, still demonstrated significant protection (Fig. 4*B*). Similarly, a biphasic curve for average tumor size confirms that the immunization with the K^b-transfected clones, even 2 days after tumor challenge, had elicited an immune response that led to the partial regression of tumors in individual mice (Fig. 4*D*).

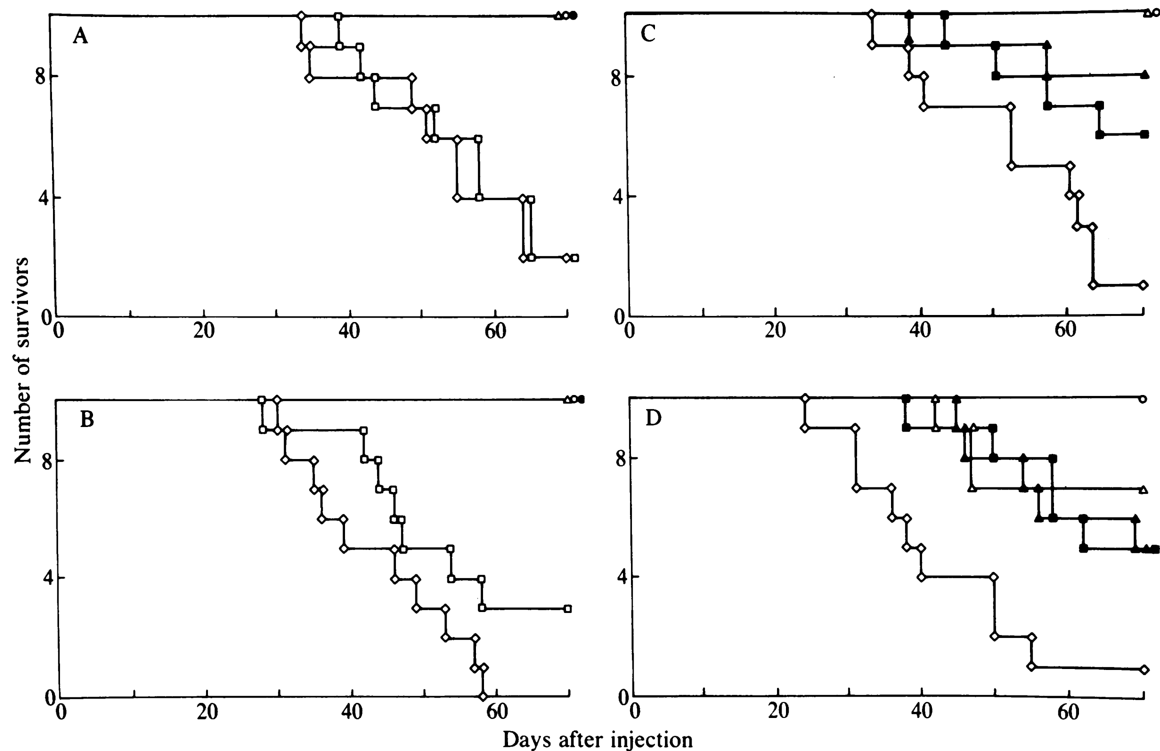

FIG. 2. Survival rates of mice injected with various doses of tumor cells. Groups of 10 mice, either C57BL/6 (*A* and *B*) or (BALB/c × C57BL/6)F_1 (*C* and *D*), were each given an intramuscular injection on the thigh of 3 × 10^6 cells (*A* and *C*) or 1 × 10^6 cells (*B* and *D*) in phosphate-buffered saline. The mice were about 6–8 weeks old at the start of the experiments. Cell-lines used were C57AT1 (□), C57AT1neo (◇), K25 (●), K73 (○), K74 (△), L99 (▲), and R9 (■).

Interestingly, the success of this immunization appears to require live cells. Irradiated cells even at a dose 3 times higher did not afford any protection (data not shown). It is conceivable that the immune presentation requires live cells. As a control for the specificity of this immunization, mice preimmunized with a nontumorigenic dose of a K^b-transfected C57AT1 clone (3 × 10^5 K73 cells) were given a tumorigenic dose of either the parental C57AT1 cells or the syngeneic BL6 melanoma cells (18). The results obtained confirmed that the protection was specific to Ad12-transformed cells and did not extend to the BL6 melanoma (data not shown).

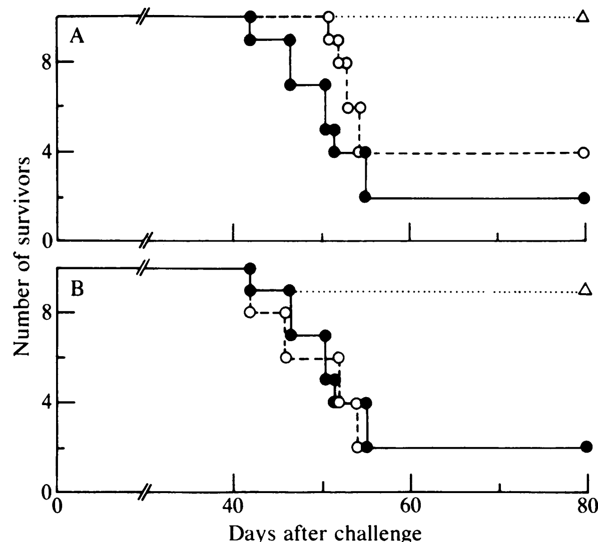

FIG. 3. Survival rates of mice immunized with IFN-treated C3AT1 cells. Groups of 10 C3H/HeJ mice were each given an intramuscular injection on the left thigh of a fixed dose of either phosphate-buffered saline (●), untreated C3AT1 cells (○), or C3AT1 cells that had been incubated for 18 hr with 2000 units of mouse IFN-α,β (△). The immunizing dose was either 2 × 10^3 cells (*A*) or 2 × 10^2 cells (*B*); both doses are below tumor threshold for these cells. Seven days later, each mouse received in the right thigh 2 × 10^6 untreated C3AT1 cells.

Since palpable tumors induced by the challenging dose of C57AT1 cells used in this study could be detected at about 5 days after injection, our ability to afford protection in mice that were immunized as late as 2 days after challenge is encouraging. It also should be pointed out that the level of expression of the exogenous K^b gene in the immunizing cells was rather low (see Fig. 1), perhaps as a result of the Ad12 function(s), which has been shown to suppress the endogenous class I genes (1, 4). Thus, the ability to induce a higher level of expression of class I antigen (e.g., with IFN treatment) may prove more effective in providing immunity against the parental tumor.

DISCUSSION

The present study suggests that the K antigen is more effective than the L antigen in the presentation of the C57AT1 tumor in syngeneic mice. We conclude that while different class I gene products may act to induce recognition of tumor cells, they do so with different efficiencies. It appears that with Ad12-transformed cells, the K and the L antigens can both act as self antigens for presentation and recognition of the Ad12 tumor antigen. We also demonstrate in this study that injection of tumor cells that have been treated with IFN or that have been transfected with a cloned class I gene may provide an effective means to potentiate the immune system to reject the parental tumor. The mechanism for this potentiation is obscure. Whether the sensitized animal now recognizes a "tumor" antigen in the context of a lower level of MHC class I antigen, or whether a heretofore unsuspected immune reaction is triggered, is presently unknown. Nevertheless, since increasing numbers of primary tumors have been found similar to Ad12-transformed cells in expressing

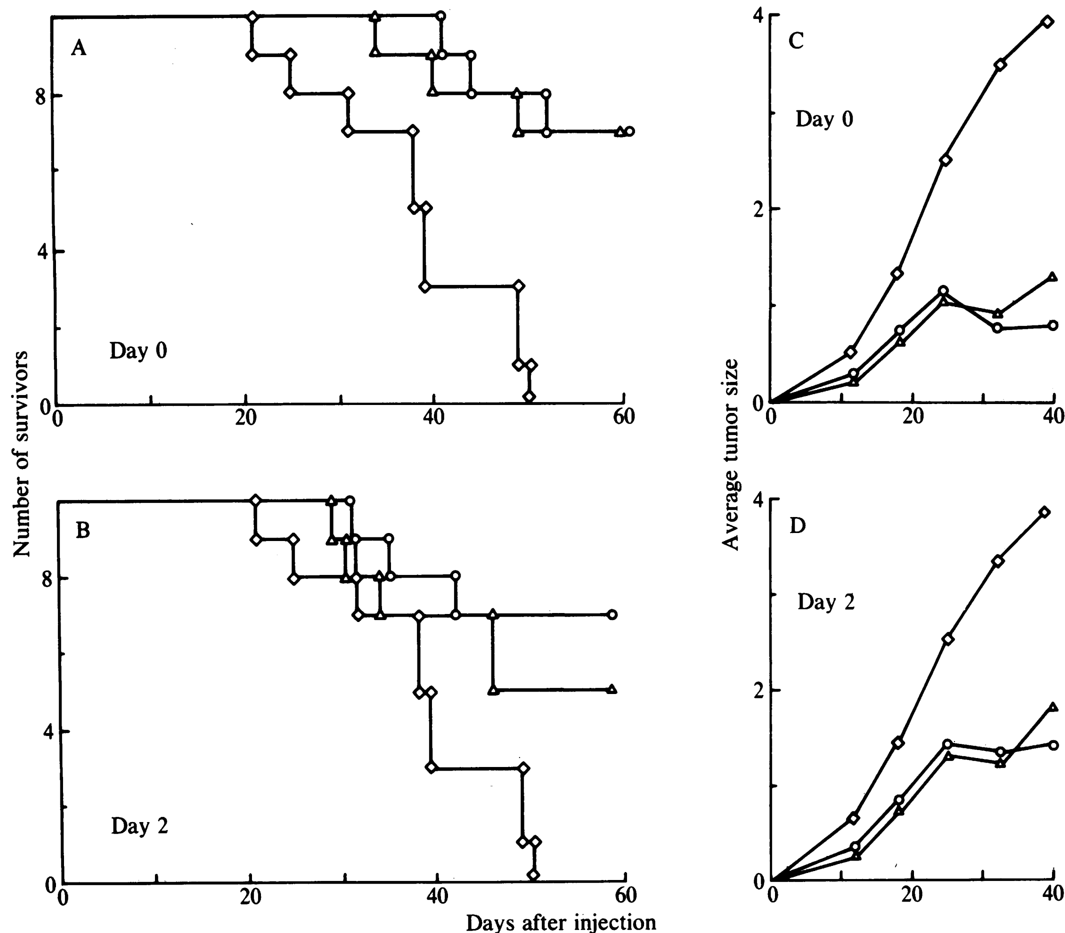

FIG. 4. Survival rates and average tumor size of mice immunized with C57AT1 cells transfected with the K^b gene. Groups of 10 C57BL/6 mice were each given an intramuscular injection on the left thigh with phosphate-buffered saline (◇), 5 × 10^5 K73 cells (○), or 5 × 10^5 K74 cells (△), followed by a second injection in the right thigh either on the same day (day 0) or 2 days later (day 2) with 8 × 10^5 C57AT1 cells. The survival rates (*A* and *B*) and the average tumor size (*C* and *D*) were determined. As the animals died during the course of the experiment because of the tumor load, their last measurements were used in all subsequent calculations of average tumor size. For that reason, the slope of the control curve is somewhat underestimated because individuals in that group began to die at a much earlier date than those in the experimental groups.

greatly reduced levels of class I antigens (19–33), the prospect of therapy by immunization with the parental tumor cells that have been manipulated in some way to induce class I gene expression offers an attractive model for further investigation.

We thank R. A. Flavell for kindly providing the cloned K^b gene, A. J. van der Eb for the Ad12 *E1A* genomic clone, and K. Ozato for the monoclonal antibody against K^b. We also thank G. F. Vande Woude for his advice and suggestions.

1. Schrier, P. I., Bernards, R., Vaessen, R. T. M. J., Houweling, A. & van der Eb, A. J. (1983) *Nature (London)* **305**, 771–775.
2. Lewis, A. M., Jr., & Cook, J. L. (1985) *Science* **227**, 15–20.
3. Zinkernagel, R. M. & Doherty, P. C. (1979) *Adv. Immunol.* **27**, 51–77.
4. Tanaka, K., Isselbacher, K. J., Khoury, G. & Jay, G. (1985) *Science* **228**, 26–30.
5. Steinmetz, M., Winoto, A., Minard, K. & Hood, L. (1982) *Cell* **28**, 489–498.
6. Weiss, E. H., Golden, L., Fahrner, K., Mellor, A. L., Devlin, J. J., Bullman, H., Tiddens, H., Bud, H. & Flavell, R. A. (1984) *Nature (London)* **310**, 650–655.
7. Hayashi, H., Tanaka, K., Jay, F., Khoury, G. & Jay, G. (1985) *Cell* **43**, 263–267.
8. Weiss, E. H., Mellor, A., Golden, L., Fahrner, K., Simpson, E., Hurst, J. & Flavell, R. A. (1983) *Nature (London)* **301**, 671–674.
9. Grosveld, F. G., Lund, T., Murray, E. J., Mellor, A. L., Dahl, H. H. M. & Flavell, R. A. (1982) *Nucleic Acids Res.* **10**, 6715–6732.
10. Graham, F. L. & van der Eb, A. J. (1973) *Virology* **52**, 456–467.
11. Cosman, D., Kress, M., Khoury, G. & Jay, G. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4947–4951.
12. Kress, M., Liu, W.-Y., Jay, E., Khoury, G. & Jay, G. (1983) *J. Biol. Chem.* **258**, 13929–13936.
13. Bernards, R., Houweling, A., Schrier, P. I., Bos, J. L. & van der Eb, A. J. (1982) *Virology* **120**, 422–432.
14. Knowles, B. B., Koncar, K., Pfizenmaier, K., Solter, D., Aden, D. P. & Trinchieri, G. (1979) *J. Immunol.* **122**, 1798–1806.
15. Boon, T. (1983) *Adv. Cancer Res.* **39**, 121–151.
16. Hui, K., Grosveld, F. & Festenstein, H. (1984) *Nature (London)* **311**, 750–752.
17. Wallich, R., Bulbuc, N., Hammerling, G. J., Katzav, S., Segal, S. & Feldman, M. (1985) *Nature (London)* **315**, 301–305.
18. Hart, I. (1979) *Am. J. Pathol.* **97**, 587–600.
19. Seigler, H. D., Kremer, W. B., Metzgar, R. S., Ward, F. E., Hwang, A. T. & Amos, D. B. (1971) *J. Natl. Cancer Inst.* **46**, 577–584.
20. Jacob, F. (1977) *Immunol. Rev.* **33**, 3–32.
21. Jones, E. A. & Bodmer, W. F. (1980) *Tissue Antigens* **16**, 195–202.
22. Trowsdale, J., Travers, P., Bodmer, W. F. & Patillo, R. A. (1980) *J. Immunol.* **130**, 2471–2478.
23. Fleming, K. A., McMichael, A., Morton, J. A., Woods, J. &

McGee, J. O. D. (1981) *J. Clin. Pathol.* **34,** 779–784.
24. Travers, P. J., Arklie, J. L., Trowsdale, J., Patillo, R. A. & Bodmer, W. F. (1982) *Natl. Cancer Inst. Monogr.* **60,** 175–180.
25. Holden, C. A., Sanderson, A. R. & MacDonald, D. M. (1983) *J. Am. Acad. Dermatol.* **9,** 867–871.
26. Lampson, L. A., Fisher, C. A. & Whelan, J. P. (1983) *J. Immunol.* **130,** 2471–2478.
27. Nanni, P., Colombo, M., DeGiovanni, C., Lollini, P., Nicoletti, G., Parmiani, G. & Prodi, G. (1983) *Immunogenetics* **10,** 361–370.
28. Natali, P. G., Giacomini, P., Bigotti, A., Lami, K., Nicotra, M. R., Ng, A. K. & Ferrone, S. (1983) *Cancer Res.* **43,** 660–668.
29. Sanderson, A. R. & Beverley, P. C. L. (1983) *Immunol. Today* **4,** 211–213.
30. Elkins, W. L., Pickard, A. & Pierson, G. R. (1984) *Cancer Immunol. Immunother.* **18,** 91–100.
31. Holden, C. A., Shaw, M., McKee, P. H., Sanderson, P. H. & MacDonald, D. M. (1984) *Arch. Dermatol.* **120,** 732–735.
32. Doyle, A., Martin, W. J., Funa, K., Gazdar, A., Carney, D., Martin, S. E., Linnoila, I., Cuttitta, F., Mulshine, J., Bunn, P. & Minna, J. (1985) *J. Exp. Med.* **161,** 1135–1151.
33. Umpleby, H. C., Heinemann, D., Symes, M. O. & Williamson, R. C. N. (1985) *J. Natl. Cancer Inst.* **74,** 1161–1168.