IMPORTANCE OF CYTOTOXIC T LYMPHOCYTES IN INFLUENZA VACCINE RESEARCH

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ABSTRACT

Influenza virus causes considerable mortality and morbidity all over the world. The antibody developed against the virus as a result of natural infection or immunization does not protect the host fully from the epidemic/pandemic caused by a virus with a change in antigenic character. This is because antibodies are directed towards surface antigens of influenza virus viz. haemagglutinin and neuraminidase which undergo antigenic change during their circulation in nature.

The virus also contains internal antigens viz. matrix protein and nucleoprotein, which are group-specific antigens. The search was made at the mechanism of immune response against influenza virus towards group specific antigens which would be cross-protective. The role of cytotoxic T lymphocytes in the cross protective immunity has been studied during the last 10 years. These studies have obvious implications for future vaccine design.

INTRODUCTION

INFLUENZA is a global health problem. It produces considerable morbidity which disrupts attendance at schools and public services. Among all diseases influenza causes maximum loss of man hours. Epidemics of influenza A and influenza B recur with monotonous frequency and each quietly and relentlessly exacts its death toll. Mortality is usually high between the two extremes of age. With the increase of geriatric population in the developed world, the importance of influenza as the underlying cause of death is considerable. With gradual improvement in the standards of hygiene in developing countries and the consequent increase in geriatric population, influenza can play a greater role in the morbidity and mortality of elderly people.

The influenza vaccine is aimed at the general population as well as high risk groups which include persons suffering from chronic debilitating diseases e.g. cardiovascular, pulmonary, metabolic disorders, and those over 45 years and particularly over 65 years of age. To minimize the impact of pandemic disease, during the interepidemic period also, vaccination has been recommended to protect the high risk groups.

Antigenic changes in the influenza viruses during the course of time and geographical locations are too well known to describe. These however, pose major problems in vaccine preparation. The antibody response to vaccine is highly specific against the viral strain employed for the manufacture of vaccine.

In vaccinated persons antibodies are generated against the surface antigens, the haemagglutinating (HA) antigen and the neuraminidase (N) antigen of the virus. These antigens are most likely to undergo antigenic shift/drift during their circulation in man and other vertebrates. The antigenic shift/drift is probably driven by antibody in the population which selects mutant viral particles with altered haemagglutinin and neuraminidase molecules. Emergence of new strains of virus is also possible through genetic recombination occurring in nature. The problem of vaccination is further complicated by simultaneous circulation of two strains of different antigenic characters[1]. Therefore humoral immune response in vaccinees immunized against a virus strain which has circulated in previous years offers at best partial protection in subsequent years.

Therefore a search was made at the mechanism of immune response in vertebrates directed towards the group-specific antigens which would be cross-protective. Besides the development of antibody towards antigen, there is another important component of immune response to viral agents, known as cell-mediated immunity (CMI).

It was demonstrated that the nucleoprotein antigen of influenza virus was highly cross-reactive in CMI. Cytotoxic T lymphocytes that are specific for cells infected by influenza A virus can be generated from the peripheral blood mononuclear cells of some immune donors. Because cytotoxic T lymphocytes show full cross-reactivity in recognition of all influenza subtypes that have been tested, these cells may confer cross-protective immunity if they play

any part in defence against infection. The present communication summarizes the work of various workers in this direction.

The lymphocytes concerned with CMI are T lymphocytes. They are now recognized as a family of different T cell subsets, which are identified by specific surface markers. Two major populations of T cell subsets in mice include the T helper cells, (TH) carrying Ly-1 surface marker and T cytotoxic (TC) cells, carrying Ly 2.3 surface markers. Helper and cytotoxic T cells do not bind free antigen. They appear to recognize antigen only in association with major histocompatibility complex (MHC) products expressed on cell membranes[2].

The MHC gene complex consists of many individual genes and although the complex as a whole performs similar functions, the arrangement of genes differs in different animal species. These genes produce many polypeptides/proteins which are expressed on the cell surface. They are classified as class I, class II and class III products. The class I products are expressed on all nucleated cells and platelets and class II products are expressed on B lymphocytes, macrophages, monocytes and activated T cells. The macrophages present the processed antigen to the T lymphocytes only in association with the MHC products, which would be either class I or class II. In other words, the T lymphocytes are restricted to either class I or class II MHC and TC (or CTL = cytotoxic T lymphocytes) require class I MHC for recognition of the antigen.

INDUCTION OF CTL DURING INFLUENZA VIRUS INFECTION/IMMUNIZATION

Initial studies on the role of CTL carried out on mice[3] showed that target cells must be histocompatible with the T cell and both the effector and target cells must possess the antigen of the class I type[4,5]: (H2 antigen of K and D series in mice). McMichael and Askonas[6] showed that CTL responses could also be elicited with cells from human peripheral blood. They also reported that human CTL responses showed considerable cross reaction between subtypes of influenza A virus. These results were confirmed by Biddison *et al*[7].

Infectious influenza virus in mice was found to be highly efficient in generating both primary and secondary CTL response *in vivo*. Earlier studies showed that inactivated influenza virus failed to stimulate a detectable CTL response even at high immunizing doses[8]. However, Greenberg *et al*[9] showed that peripheral blood lymphocytes from volunteers inoculated with inactivated influenza vaccine as well as with live virus, showed cytotoxicity against influenza virus infected cells *in vitro*.

Ennis *et al*[10] further confirmed that it is not necessary to use live virus vaccine to generate CTL in man. HLA restricted virus specific CTL responses are produced against live as well as inactivated influenza vaccines[10]. McMichael *et al*[11] inoculated human volunteers, intranasally with A/Munich/1/79 (H1N1) strain of influenza virus. All subjects with demonstrable T cell responses cleared the virus effectively. This response was observed even in volunteers who did not have specific antibody. It was concluded that T cells play a significant part in recovery from influenza virus infection[11].

SPECIFICITY OF ANTIGEN RECOGNITION BY CTL

Influenza viruses are excellent tools for detailed analysis of antigen specificity in CTL response because they are well-defined in molecular terms. In other words, the structures of the viral components of natural variants or genetic recombinants are known. HA and NA are strain-specific antigens, present on the surface of the virion. The matrix protein (M) and nucleoprotein (NP) antigens are type-specific antigens for influenza type A virus which do not cross-react serologically with influenza type B virus. However M and NP antigens show cross reaction between strains of the same type.

Generation of cross reactive and virus strain-specific CTL was studied in influenza virus infection or immunization by several workers[5,12–14]. A majority of the cytotoxic T cells generated by immunization with any influenza A were highly lethal for a cell infected with any other strain of influenza A virus, but not to a cell infected with a B type virus. It was reported that cytotoxicity was directed against the shared matrix protein, which is now known to be present on the infected cell surface used as a target[15,16]. A small number of influenza immune CTL are apparently haemagglutinin-specific. Secondary stimulation *in vitro* with isolated haemagglutinin or inactivated virus leads to the emergence of the virus-specific T cell subsets[12,13].

Recently, Townsend and McMichael[17] reported that infection with influenza virus induces CTL response that are usually cross-reactive with influenza virus A subtype but a minor population of CTL was strain-specific[17].

PROTECTIVE EFFECT OF CTL AGAINST INFLUENZA

It is established that CTL may be of significance in the development of resistance against infection. Ennis *et al*[18] found that CTL took part in the defence of the lung against infection with influenza virus. Yap *et al*[19] reported that adoptive transfer of CTL would protect mice against lethal dose of virus inoculated intranasally.

Websee and Askonas[20] established a good correlation between level of memory CTL and cross protection against different type A influenza viruses *in vivo*. T cells from primed mice led to a reduction of replication of homologous virus in the recipient mice on adoptive transfer. Protection only occurred when donor cells and recipient mice shared the K and D end of H-2 complex. It implied that CTL might be effector cells. The effect was abolished by treatment with anti Ly-2 serum and complement. However this type of evidence for protection of mice by T cells was thought to be merely circumstantial in view of the complex reactions of T cell subsets in lymphoid organs. Therefore, testing of the effect, of CTL clones on virus replication *in vivo* after influenza virus infection was required.

DEVELOPMENT OF CYTOTOXIC T LYMPHOCYTE CLONES RESTRICTED BY MHC CLASS I AGAINST INFLUENZA VIRUS

Braciale *et al*[21] generated *in vitro* continuous lines of murine CTL directed to A/Jap/57 (H2N2) influenza virus. All CTL lines were restricted by MHC class I antigens and exhibited Ly-2 surface antigen marker. Among the 12 lines stimulated and selected there were CTL clones of the following types. (i) Strain-specific CTL lines were cytotoxic exclusively for A/Jap/57 (H2N2) virus infected cells; (ii) Subtype specific CTL lines, and (iii) Cross-reactive CTL lines exhibiting cytotoxicity on all type A subtypes.

CTL CLONES RESTRICTED BY MHC CLASS II ANTIGENS

As described earlier[22], classically-described antiviral CTL are restricted by class I MHC gene products in their recognition of viral and other foreign antigens. Although alloreactive CTL responses to MHC class II antigens were documented since 1975, T cells restricted by MHC class II molecules in antigen recognition and having surface markers Ly-1 similar to TH cells have only been recently appreciated as distinct T lymphocyte population.

Kaplan *et al*[23] analysed clones from the pool of human CTL effectors. They revealed CTL clones which possessed T4/Leu 2 surface markers and were restricted by MHC class II products. These clones were similar to those described in mice cross-reactive, subtype-specific and strain-specific.

These clones produced factors like interleukin-2 which allowed them to proliferate in the absence of exogenous lymphokines.

This work on CTL clones revealed that besides classically described antiviral CTL restricted by class I MHC gene products, CTL restricted by MHC class II molecules also exist. Such CTL clones, restricted by class II HLA antigens have been described against herpes simplex virus[24], measles[25] and Epstein-Barr virus[26].

PROTECTIVE EFFECT OF T CELL CLONES *IN VIVO*

Lin and Askonas[27] used the cytotoxic CTL clone viz. L4 of BALB/c origin developed from A/X-31 (H3N2) infected lymphoblast for studying the protective effect. This clone could grow continuously in the presence of T cell growth factor(s), carried Ly-2 markers and killed the target cells with K and D region restriction.

Intranasal infection of sublethally irradiated BALB/c with 20–30 haemagglutinin units of A/X-31 virus leads to death within 19 days. After transfer of 3×10^6 L4 cells, three out of four mice survived for 4 months.

BALB/c mice were infected intranasally with A/USSR/90/77 (H1N1) influenza virus, 24 h later, L4 cells were transferred i/v into the infected mice. Three days later the lungs were removed and the virus titre (HA) was assayed. The lung titre in the mice receiving L4 cells was three orders of magnitude lower than in control mice that received virus alone.

Taylor and Askonas[28] reported a murine CTL clone, T5/5 which was different morphologically and in *in vivo* migration pattern, from the L4 clone. In adoptive transfer experiments, the clone T5/5 localized in the lung but did not limit virus replication in contrast to clone L4, although they efficiently lysed influenza infected target cells *in vitro*. The clone T5/5 did not release significant amount of immune interferon on contact with influenza virus infected target cells. CTL clone L4 regularly released IFN

within 6 h after contact with a target cell it recognizes[29].

The above reports[28,29] document the secretion of lymphokines most notably interferon by CTL population. It was thought that these lymphokines might serve as a primary antiviral effector mechanism *in vivo*. Thus, the relative importance of cytolysis by CTL and soluble factors (viz. interferon) released by CTL as antiviral effector mechanism was questioned.

Lukacher *et al*[30] examined the *in vivo* antiviral effector activity of several cloned CTL[30]. One CTL clone, which recognized only type A influenza viruses of the H2N2 subtype *in vitro*, selectively promoted recovery from lethal influenza infection in a subtype specific way. In contrast, the cross-reactive CTL likewise protected mice infected by either of two different virus subtypes. When mice were infected with two different subtypes, the subtype specific CTL clone did not promote recovery but the clone could only reduce the virus titre of the strain representation of the subtype recognized by the CTL clone. Their work favoured the concept that CTL express their antiviral effect *in vivo* by direct cytolysis of infected cells.

Thus, the important function of CTL in influenza infection was demonstrated with the finding that murine CTL clones could limit virus replication *in vivo*.

INFLUENZA VIRAL COMPONENTS RECOGNIZED BY CTL

Once it was known that a majority of the CTL induced by a strain of influenza virus would cross-react with other strains of the same type it became necessary to identify the viral components (viral antigens) present in different strains which would be recognized by cross-reactive CTL.

Townsend *et al*[31], using L cells expressing either nucleoprotein or haemagglutinin by DNA-mediated gene transfer showed that nucleoprotein is the major target for CTL that are cross-reactive. Recombinant vaccinia virus engineered to express influenza NP also has been used to demonstrate that murine CTL recognize the NP molecule[32]. The nucleoprotein is not glycosylated; it accumulates in nuclei of infected and transfected cells and has none of the sequence characteristics of an integral membrane protein. This raised the question of how NP is transported to the plasma membrane where CTL recognition is assumed to occur. To investigate the mechanism by which this nonglyco protein component of the virus was recognized by CTL, a series of deletion mutants of influenza A virus NP genes were studied. The results showed that CTL recognize three distinct epitopes of the NP molecule. Both N and C terminal fragments of the protein are transported, independently of each other, to the site of recognition by CTL[33]. It has been shown that not only mouse but also human CTL from several but not all donors respond to nucleoprotein[34].

In view of the possibility that purified NP may have potential as a vaccine[35], its relevance as a target antigen in productive influenza infection in the lung or trachea of infected mice was investigated[36]. Mice were infected with influenza virus and CTL clones were injected i/v, 1–2 h later. Several influenza NP specific T cell clones were transferred into syngeneic mice infected with lethal/sublethal doses of different A type viruses. The study demonstrated that NP specific CTL clones can limit influenza virus replication *in vivo* and also protect against lethal infection. Thus, NP specific CTL clones limit virus replication *in vivo* and hosts primed with purified NP were protected against lethal infection. NP thus becomes a candidate vaccine.

Epitopes of nucleoprotein recognized by CTL in association with class I molecules of the MHC in both mouse and man were defined as short synthetic peptides derived from the NP sequence[37]. These results offered new ways of stimulating CMI with vaccines. However, T cell recognition of peptides in conjugation with MHC molecules is influenced by genetic differences. A given peptide is unlikely to be of general value for stimulating CMI in a polymorphic population.

RECOGNITION OF HEAT INACTIVATED INFLUENZA VIRUS BY CTL CLONES

Townsend *et al*[33] reported that nucleoprotein antigen can serve as target antigen for class I restricted CTL. In other words, class I restricted CTL can recognize noninfectious antigen. Hosaka *et al*[38] found that influenza virus heat inactivated at 55°C for 30 min retained fusion activity and the capabilities to elicit CTL and to sensitize target cells for lysis by CTL. They have also reported differences in patterns of recognition of targets and of protection of mice with class I and class II restricted CTL clones. Class I restricted clones recognized target cells sensitized by a wide range of dose of the inactivated virus; they recognized NP protein antigen, whereas class II restricted CTL recognized HA antigens.

Cross-reactive class I restricted clones protected mice from lethal virus infection, while class II restricted clones did not, but rather tended to shorten the period of survival.

Preliminary evidence suggests that high levels of TH cells class II restricted CTL can be harmful to the host. TH clones, that are specific to HA, enhance replication of influenza virus in lungs and induce lung consolidation.

It was suggested[37] that non-membrane viral proteins are degraded in the cytoplasm of the infected cell, producing short denatured peptides that are exposed from the cell by some unknown mechanisms. Such degraded viral proteins may then become available for recognition by CTL in association with class II MHC molecules in a way similar to that which helper T cells recognize denatured or degraded proteins with MHC class II molecules.

Morrison *et al*[39] were of the opinion that both class I and class II MHC restricted CTL would recognize target cells exposed to infectious virus. However, target cells exposed to exogenously introduced virion would also be recognized by MHC class II restricted CTL provided the antigens are processed by lysosomal vesicles.

The above observations suggest that class II restricted CTL are likely to play a positive role in viral clearance and recovery from influenza viral infection. Activation of these class II restricted TH cells, while responsible for removal of virus-infected cells, can also induce immunopathology as mentioned above and vaccines should ensure a balanced immune response.

Further studies on the induction of class I and class II restricted CTL by inactivated vaccine in humans and also on the role of memory CTL are necessary.

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1. Leading articles, *Br. Med. J.*, 1978, **2,** 229.
2. Roitt, I., Brostoff, J. and Male, D., *Immunology*, 1985, **11,** 1.
3. Yap, K. L. and Ada, G. L., *Immunology*, 1977, **32,** 151.
4. Ennis, F. A., Martin, W. J., Verbonitz, M. W. and Butchko, G. M., *Proc. Natl. Acad. Sci. (USA)*, 1977, **74,** 3006.
5. Doherty, P. C., Solter, D. and Knowles, B. B., *Nature (London)*, 1977, **266,** 361.
6. McMichael, A. J. and Askonas, B. A., *Eur. J. Immunol.*, 1978, **8,** 705.
7. Biddison, W. E., Shaw, S. and Nelson, D. L., *J. Immunol.*, 1979, **122,** 660.
8. Braciale, T. J. and Yap, K. L., *J. Exp. Med.*, 1978, **147,** 1236.
9. Greenberg, S. B., Criswell, B. S., Six, H. R. and Couch, R. B., *Infect. Immun.*, 1978, **20,** 640.
10. Ennis, F. A. *et al.*, *Lancet*, 1981, **2,** 887.
11. McMichael, A. J., Gotch, F. M., Noble, G. R. and Beare, A. S., *New Engl. J. Med.*, 1983, **309,** 13.
12. Zweerink, H. J., Courtneidge, S. A., Skehel, J. J., Crumpton, M. J. and Askonas, B. A., *Nature (London)*, 1977, **267,** 354.
13. Effros, R. B., Doherty, P. C., Gerhard, W. and Bennink, J., *J. Exp. Med.*, 1977, **145,** 557.
14. Braciale, T. J., *Cell. Immunol.*, 1977, **33,** 423.
15. Braciale, T. J., *J. Exp. Med.*, 1977, **146,** 673.
16. Ada, G. L. and Yap, K. L., *Immunochemistry*, 1977, **14,** 643.
17. Townsend, A. R. M. and McMichael, A. J., *Prog. Allergy*, 1985, **36,** 10.
18. Ennis, F. A., Wells, M. A., Butchko, G. M. and Albrecht, P., *J. Exp. Med.*, 1978, **148,** 1241.
19. Yap, K. L., Ada, G. L. and Mckenzie, I. F. C., *Nature (London)*, 1978, **273,** 238.
20. Websee, R. G. and Askonas, B. A., *Eur. J. Immunol.*, 1980, **10,** 396.
21. Braciale, T. J., Andrew, M. E. and Braciale, V. L., *J. Exp. Med.*, 1981, **153,** 910.
22. Zinkernagel, R. M. and Doherty, P. C., *Adv. Immunol.*, 1979, **27,** 51.
23. Kaplan, D. R., Griffith, R., Braciale, V. L. and Braciale, T. J., *Cell Immunol.*, 1984, **88,** 193.
24. Yasukawa, M. and Zarling, J. M., *J. Immunol.*, 1984, **133,** 422.
25. Jacobson, S., Richert, J. R., Biddison, W. E., Satinsky, A., Hartzman, R. J. and Mcfarland, H. F., *J. Immunol.*, 1984, **133,** 754.
26. Meuer, S. C., Hodgdon, J. C., Cooper, D. A., Hussey, R. E., Fitzgerald, K. A., Schlossman, S. F. and Reinherz, E. L., *J. Immunol.*, 1983, **131,** 186.
27. Lin yun-Lu and Askonas, B. A., *J. Exp. Med.*, 1981, **154,** 225.
28. Taylor, P. M. and Askonas, B. A., *Eur. J. Immunol.*, 1983, **13,** 707.
29. Morris, A. J., Lin yun-Lu and Askonas, B. A., *Nature (London)*, 1982, **295,** 150.

30. Lukacher, A. E., Braciale, V. L. and Braciale, T. J., *J. Exp. Med.*, 1984, **160**, 814.
31. Townsend, A. R. M., McMichael, A. J., Carter, N. P., Huddleston, J. A. and Brownless, G. G., *Cell*, 1984, **39**, 13.
32. Yewdell, J. W., Bennink, J. R., Smith, G. L. and Moss, B., *Proc. Natl. Acad. Sci.* (USA), 1985, **82**, 1785.
33. Townsend, A. R. M., Gotch, F. M. and Davey, J., *Cell*, 1985, **42**, 457.
34. McMichael, A. J., Michie, C. A., Gotch, F. M., Smith, G. L. and Moss, B., *J. Gen. Virol.*, 1986, **67**, 719.
35. Wraith, D. C. and Askonas, B. A., *J. Gen. Virol.*, 1985, **66**, 1327.
36. Taylor, P. M. and Askonas, B. A., *Immunology*, 1986, **58**, 417.
37. Townsend, A. R. M., Rothbard, J., Gotch, F. M., Bahadur, G., Wraith, D. and McMichael, A. J., *Cell*, 1986, **44**, 959.
38. Hosaka, Y. *et al.*, *Proc. VII Int. Congr. Virol.*, Edmonton, Canada, 9–14 August, 1987.
39. Morrison, L. A., Lukacher, A. E., Braciale, V. L., Fan, D. P. and Braciale, T. J., *J. Exp. Med.*, 1986, **163**, 903.

NEWS

HEPARIN STOPS THE CLOTS

When blood comes into contact with an alien surface, it rapidly forms a clot. This is why so much effort has been devoted to finding inert or biocompatible plastics for 'spare-part' surgery, and it is a major problem with dialysis and heart-lung machines where the patient's blood contacts metal surfaces. To prevent potentially fatal thromboses while on one of these machines, the patient must be given anticoagulants — but these can lead to equally dangerous haemorrhages.

Now a Scandinavian team has made a breakthrough that could get round the problem by making the machine's surface blood-compatible. The normal aim is to make the surfaces as inert as possible, but Professor Per Olsson of the Karolinska Hospital in Stockholm looked at it another way — what keeps blood flowing in normal arteries and veins? The answer is heparin, a polysaccharide found in the walls of blood vessels, which acts as an immobilised anticoagulant. It is a well-known compound and it has long been used as an anticoagulant drug for post-operative patients.

Olle Larm, the carbohydrate chemist in Professor Olsson's team, found a way to bind heparin to surfaces without altering its effectiveness. The result is a biocompatible metal or plastic. Carmeda, part of the Norwegian Norsk Hydro group, holds the patent on the process.

Heparin-coated materials could be used in a wide range of blood-contacting medical devices such as sensors, catheters, pumps, oxygenators and artificial organs. The Karolinska Hospital already has a heparin-coated heart-lung machine that has saved the lives of two lung-damaged patients, and the St. Goran Hospital is developing a smaller machine specifically to help new-born babies with underdeveloped lungs. Olsson believes that heparin-coated equipment will be common place in major surgery in the 1990s. (*Chemistry in Britain*, March 1988, p. 203, Published by the Royal Society of Chemistry, Burlington House, London WC1B OBW).