CELLULAR IMMUNE RECOGNITION OF INFLUENZA A VIRUSES IN EQUINES: *IN VITRO* AND *IN VIVO* STUDIES ON THE IMMUNOGENICITY OF EQUINE INFLUENZA VIRUSES

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Abstract

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In vitro and *in vivo* studies on immunogenicity of equine influenza viruses were investigated in an equine model using the cell proliferation assay. Proliferating cells were characterized and the induction of immunogenicity in these cells was also determined. Both equine-1 (H7N7) and equine-2(H3N8) influenza viruses were found to be mitogenic *in vitro* but lymphocyte responsiveness was more marked with the former. Lymphocyte responsiveness was also observed *in vivo* from 72 hours up to 6 weeks following intravenous injection of experimental horses with influenza viral glycoproteins while haemagglutination inhibition and protective neutralising antibodies showed that equine influenza viruses and their surface glycoproteins could induce B and T cell responses in horses. This induction occurred through direct binding of the virus surface antigens to receptors on the surface in addition to previously reported activation of class I and class II major histocompatibility complex restricted T cells. The proliferating cells produced immunoglobulin isotypes IgGa, IgGb, IgGc and IgG(T). The role of memory B and T cells in the protection of immunized animals is highlighted.

Mitogenicity, viral glycoproteins, lymphoproliferation, immunoglobulin isotypes

Lymphocyte mitogens have been widely used to study events involved in activation and differentiation of B and T lymphocytes in rodents and man (Anders et al. 1984). Viruses, such as vesicular stomatitis virus (Goodman-Snikkof and McSharry 1980), Sendai virus (Kizaka et al. 1983) and some strains of human influenza A, B and recombinant viruses (Butchko et al. 1978; Armstrong et al. 1981; Anders et al. 1984) have been shown to be mitogenic. While these earlier workers used normal or primed spleen cells from human and murine models *in vitro*, there is a dearth of information on the *in vivo* immunogenic properties of equine influenza viruses. It is well known that infection or immunization with influenza viruses or vaccines results in production of antibodies directed against various components of the virus. However, it is not clear how the mitogenicity and immunogenicity are induced *in vivo* by viral glycoproteins. The present paper therefore addresses immunological issues such as the *in vitro* and *in vivo* antigenicity and immunogenicity of equine influenza virus glycoproteins in an equine model.

Materials and Methods

Viruses and glycoproteins

The viruses used in these studies were equine-2 viruses recently isolated from Nigeria, A/Eq/Ibadan/4/91, A/Eq/Ibadan/6/91 and A/Eq/Ibadan/9/91 (H3N8) (Adeyefa and McCauley 1994), the prototype equine-2 virus, A/Eq/Miami/63 (H3N8) and the prototype equine-1 virus, A/Eq/Prague/56 (H7N7) designated Ib4, Ib6, Ib9, M63 and P56, respectively. The viruses were grown in 11-day old embryonated chicken eggs and purified by

differential sedimentation through 15-60% sucrose gradients. The haemagglutinin (HA) and neuraminidase (NA) glycoproteins were isolated as previously described by Brand and Skehel (1972). Briefly, the purified viruses were treated with bromelain (Sigma Corporation, USA) and the solubilised glycoproteins were isolated by centrifugation in 30 - 60% sucrose gradients. The glycoproteins were pooled and concentrated by vacuum dialysis and the concentration was determined by the method of Bradford (1976). The preparations were stored at -2 °C until used.

Peripheral Blood Lymphocytes

Peripheral blood lymphocytes (PBL) were isolated from two influenza naive foals as described by Adeyefa (1989). Briefly, 40 ml heparinized blood was collected aseptically by jugular venipuncture and mononuclear cells were obtained from the blood by density gradient centrifugation on Lymphocyte Separating Medium (Flow Laboratories, Scotland). The isolated mononuclear cells were washed thrice in Minimum Essential Medium (MEM) and resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 containing 10% foetal calf serum. Viability test on separated lymphocytes was done by trypan blue exclusion method with percentage viability ranging from 89.8 to 99.5%. 200 µl of cultures containing 2×10^5 viable cells/ml from each foal were prepared in triplicates in flat-bottomed 96-well micro-culture plates.

Lymphocyte proliferation assays

The glycoprotein preparations were thawed on ice and diluted in PBS to concentrations ranging from 0.1 to 200 µg/ml and used in 20 µl volumes to stimulate the lymphocytes. Mitogenic lectins such as Concanavallin A(ConA), phytohaemagglutinin (PHA) and poke-weed mitogen (PWM) at concentrations of 0.5 µg, 1 µg and 5 µg/ml, respectively, were used as positive control antigens in 20 µl volumes. The cell proliferation assay was used based on the method described by Mosmann (1983) with slight modification. Briefly, 20 µl of varying concentrations of the test glycoprotein antigens and the lectins were pipetted into the wells of the micro-culture plates in triplicates to which were then added 200 µl of cell culture containing 2×105 viable cells/well to be stimulated by the antigens. Cultures containing neither antigens nor lectins but PBS in triplicates served as unstimulated controls. The plates were then incubated at 37 °C for 72-96 hours in a 5% CO_2 incubator.

Lymphocyte proliferation was measured by adding 15 µl of 50 mg/ml MTT solution (2-(4,5-diamethylthiazol-2-yl) 2, 5 diphenyl tetrazolium bromide) to culture wells and incubating the plates for 4 hours at 37 °C in a 5% CO_2 incubator. This allowed the tetrazolium salt to be converted to a blue formazan to quantitate the total cellular protein as being directly proportional to the number of proliferating cells measured as absorbance values with an ELISA plate reader (Riss 1991). This was followed by addition of 100 µl/well of dimethyl formamide (Sigma), shaking the plates on an orbital shaker and incubating them for another 1 hour as before. The plates were then shaken for about 5 minutes and the absorbance at 570 nm wavelength was measured in a Dynatech MR 5000 ELISA plate reader with a 630 nm filter which eliminates or significantly reduced background absorbance from cell debris, finger prints and other non-specific absorbance. The results were expressed as stimulation indices,

$$(SI) = \frac{\text{Absorbance values of stimulated cultures}}{\text{absorbance values of unstimulated culture}}$$

In vivo Studies

Three animals comprising a yearling colt, a 6-month-old foal and a 4-month-old male donkey were used as test animals while two adult female donkeys served as control animals. Sera from each animal had been previously tested twice for influenza virus antibodies and found to be negative on both occasions. Two aliquots of heparinized blood were then aseptically collected from the jugular vein of each animal into sterile 50 ml centrifuge tubes. From one aliquot, PBL were obtained from 40 ml of blood for each animal as described above while the other aliquot was stored at 4 °C. Each of the 3 test animals was then injected intravenously (iv) with 5ml of the respective Ib6, M63 and P56 virus glycoproteins at concentrations of 10 µg/ml. The separated lymphocytes were assayed as unstimulated culture by non-radioactive cell proliferation assay in sterile 96-well flat bottomed tissue culture plates as described above. Seventy-two hours post injection, 40 ml heparinised blood were collected from each test animal and the lymphocytes obtained were assayed as stimulated cultures. PBL from the aliquots stored at 4 °C were also assayed simultaneously as unstimulated cultures. Subsequently, 40 ml of venous blood were collected from each animal at 1, 2, 4 and 6 weeks and the PBL obtained were assayed as stimulated cultures while the PBL obtained from the control donkeys served as unstimulated control cultures. The assayed plates were read spectrophotometrically with the Dynatech MR 500 ELISA plate reader at 570 nm wavelength and 630 nm reference filter as above.

Allantoic fluid virus antigen as well as intact and denatured virus glycoproteins were used to induce lymphocyte proliferation *in vitro* and *in vivo*. Denaturation was achieved by heating the glycoproteins at 90 °C for 2 minutes for *in vivo* lymphocyte stimulation and by adding 1% sodium dodecyl sulphate for *in vitro* stimulation. Five ml of 10 µg/ml of intact and denatured glycoproteins were injected iv into each of two influenza-naive donkeys from which PBL were obtained post injection. Lymphocyte proliferation assays were then carried out on both *in vitro*

and *in vivo* cultures. In addition to the negative control well with PBS. supernatant in 20 µl volume from mock-infected BHK monolayer cells and 5 µg volume ovalbumin were used to stimulate the *in vitro* cultures of lymphocytes in triplicate wells.

In another experiment, 5 µl each of monoclonal antibodies (MAbs) H1, H3, H4 and H6 against A/Eq/Miami/63 (H3N8) and 5 µl each of potassium metaperiodate-treated polyclonal antisera raised against A/Eq/Fontainebleau/79 (H3N8), A/Eq/Newmarket/76 (H3N8) and normal goat serum were added to triplicate wells of PBL at 2×10^5 cells/ml. The cells were then stimulated with 100 µg of A/Eq/Ibadan/6/91 (H3N8) virus glycoproteins and lymphocyte proliferation assay was then carried out as above. In the present experiment, two of the 3 previously immunized animals (now aged 15 and 17 months, respectively, about 11 months after the previous challenge) and an adult influenza-naive donkey were used as test animals while another adult influenza-naive donkey served as unchallenged control. Sera from the 4 animals were tested for neutralizing antibodies against influenza virus. Titers of < 80 were observed in the 2 previously challenged animals and <10 in the 2 influenza-naive animals. The 3 test animals were then challenged intranasally with 5 ml of allantoic fluid virus antigen (10^9EID_{50}) of A/Eq/Ibadan/6/91, (H3N8) and observed for 28 days.

Serum was collected from each animal at 7 and 21 days post challenge. Serial two-fold dilutions of each serum and a negative control goat serum were reacted in neutralization tests in a 96-well flat bottomed tissue culture plate with 50 µl (10^9EID_{50}) homologous allantoic fluid virus using chicken embryo fibroblast monolayer cells incubated at 37 °C in 5%CO_2 incubator for 72 hours and checked for cytopathic effect (CPE) or virus neutralization.

Serology

Sera were obtained from test and control animals at the same time the blood for cell proliferation assay was collected and also at 12 weeks after stimulation. Haemagglutination titrations of the viruses and HI tests with the harvested sera were performed in microtiter plates with 1% chicken red blood cells by standard methods using allantoic fluid virus antigens and the virus glycoproteins. Antisera raised in rabbits against A/Equine/Fontainebleau/79, (H3N8) and A/Equine/Prague/1/56 (H7N7) were used as positive control sera while serum from a pathogen-free guinea pig served as negative control serum. All the sera used in the HI tests were treated with potassium M-periodate (Sigma) to remove any inhibitors present in normal serum and thus preclude non-specific haemagglutination inhibition.

Serial two-fold dilutions of the test and control sera were also reacted in neutralization (N) tests in 96-well flat bottom tissue culture plates with allantoic fluid virus antigens containing appropriately 10^9EID_{50} using Vero monolayer cells incubated at 37 °C for 48 hours and checked for CPE and/or neutralization.

HI titers were expressed as the reciprocal of the highest serum solution inhibiting 4 HA units of the viruses while the neutralization titers were expressed as the reciprocal of the highest serum dilution neutralizing 10^9EID_{50} of the viruses. For the HI and N tests A/Equine/Ibadan/4/91 (H3N8) was used as positive control antigen.

Experimental challenge with live viruses

All the animals were clinically examined twice a week for a further 3 months for signs of influenza and other viral respiratory diseases. At 6 months post injection each of the 3 test animals was challenged with an intranasal inoculation of allantoic fluid virus antigens containing approximately 10^9EID_{50} of the respective virus. One control donkey was challenged with A/Equine/Ibadan/4/91 virus while the other served as uninfected control. The animals were then housed in insect-proof locked up isolation boxes and clinically examined for 3 weeks for signs of influenza infection. Blood was collected for sera from each animal on days 5 and 14 post infection. A 1:10 dilution of nasopharyngeal washings from each animal was inoculated into BHK monolayers cells in 25 ml tissue culture flasks incubated at 37 °C for about 7 days for virus recovery. HA tests were performed on tissue culture supernatant which was then passaged thrice.

Characterisation of proliferation cells

PBL were obtained as described earlier. The cells were resuspended at concentration of 1×10^6 cells/ml in RPMI 1640 medium containing 2% goat serum. 200 µl of cell suspension at 2×10^5 cells/ml in triplicate wells were stimulated with 100 µg each of A/Eq/Ibadan/6/91 (H3N8). A/Eq/Prague/56 (H7N7) virus glycoproteins as well as 1 µg PHA and 0.5 µg Con A in 20 µl volumes and incubated at 37 °C in a 5% CO_2 incubator for 72 hrs. Ten ml of resuspended cells were passed over nylon wool thrice to deplete them of B cells to the barest minimum (Anders et. al (1984) found that two passages over nylon wool reduced B cell population to < 1.4%). 200 µl of T-cell enriched suspension of 2×10^5 cells/ml were then stimulated as above. Two sets of triplicate wells of cells with only PBS and culture supernatant from mock-infected BHK monolayer cells served as unstimulated control cultures. Lymphocyte proliferation assays were performed and the results expressed as above. It was not possible to determine T cell subsets or their lineages.

Determination of concentration of isotypes of immunoglobulins (Igs) produced by the proliferation cells

Two ml of PBL at 2×10^5 cells/ml were stimulated as above in 24-well tissue culture plates incubated at 37 °C in

5% CO_2 incubator for 72 hours after which 48 ml of tissue culture supernatants were harvested, pooled and concentrated to 5 ml by vacuum dialysis. Total Ig concentration was measured with a Philips scan UV Spectrophotometer. The Igs were then fractionated on a DEAE-Sephadex A25 ion-exchange chromatography column with a stepwise gradient of 0.01M-0.75M Tris HCL buffer pH 8.6 + 0.5M NaCl at 4 °C at a flow rate of 1ml/min and fraction size of 5 ml. The optical density (OD) of eluates was monitored at 280 nm wavelength. Similarly, 5 ml of unstimulated cell culture supernatant were also subjected to ion-exchange chromatography as above. OD values were fed into a computer to plot the Ig isotypes' peaks using the Freelance 3.01 option programme.

Results

Fig.1 shows the dose response curves of equine PBL against the glycoproteins of equine-1 (H7N7) and equine-2 (H3N8) influenza A viruses used in this study in 72 hrs cultures *in vitro*. Maximum stimulation occurred at concentrations between 50 and 70 µg/ml in H3N8 viruses and between 75 and 100 µg/ml in the H7N7 virus. A plateau was also observed in lymphocyte responsiveness at concentrations between 50 and 100 µg/ml in both H3N8 and H7N7 viruses. Lymphocyte reactivity was more marked in Ib4 virus followed by Ib6 and M63 viruses which gave slightly similar responsiveness while it was much less in Ib9 virus. On the other hand, there was a steady increase in lymphocyte responsiveness to P56 virus beyond 100 µg/ml before dropping. Lymphocyte reactivity was more pronounced with the lectins used as positive control mitogen while there was little or no proliferation in the PBS negative control cultures. The 96 h cultures gave similar dose response curves but with much lower SI (data not shown).

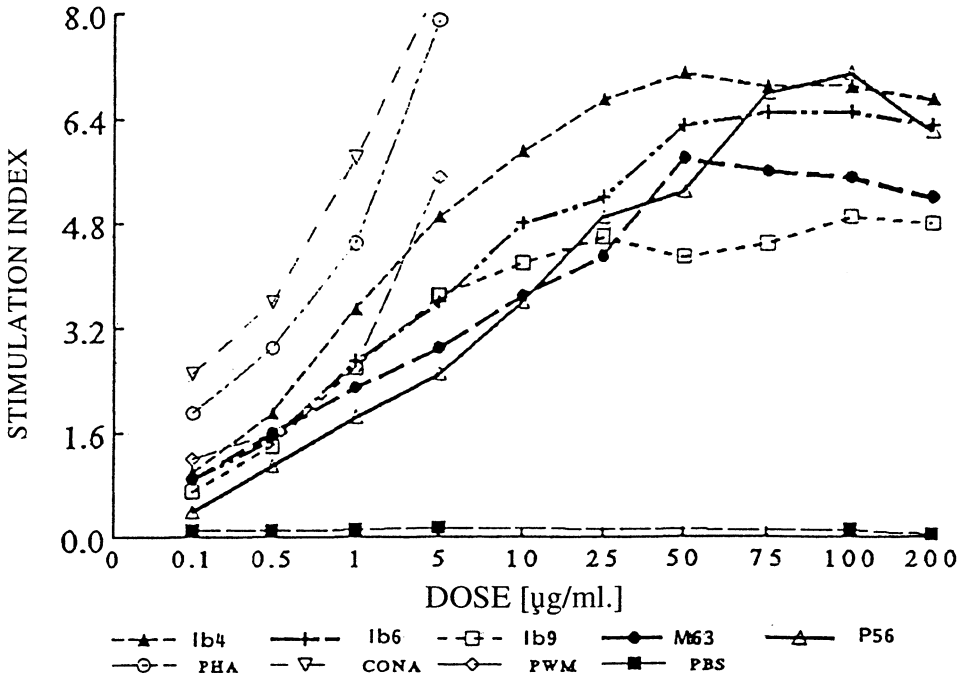

Fig. 1: Dose response curve of equine peripheral lymphocyte cultures

Lymphocyte responsiveness to the glycoproteins of each virus *in vivo* is shown in Fig. 2. The glycoproteins induced PBL proliferation from 72 h up to 6 weeks post iv injection. Maximum proliferation was observed at 72 h with SI of 9.16 for Ib6, 7.88 for M63 and 8.62 for P56. The SI slightly less for each virus at 1 week with P56 giving a better responsiveness than Ib6 and M63. Lymphocyte responsiveness decreased significantly ($P < 0.001$) by about 45-66% in all cases from 2-6 weeks but there was no significant ($P > 0.05$) difference in individual responsiveness during each period. Infectious virus as well as intact and denatured glycoproteins induced lymphocyte proliferation *in vitro* and *in vivo* as indicated by SI shown in Fig. 3. There were no significant differeces in SI obtained in *in vitro* and *in vivo* cultures. Lymphocyte proliferation was inhibited by the MAbs and polyclonal antisera added to stimulated cultures while proliferation was observed in wells to which normal goat serum was added. The implications of these results are that the mitogenic and immunogenic activities of equine influenza virus glycoproteins appear to be induced by direct binding of the protein antigens to receptors on the cell surface.

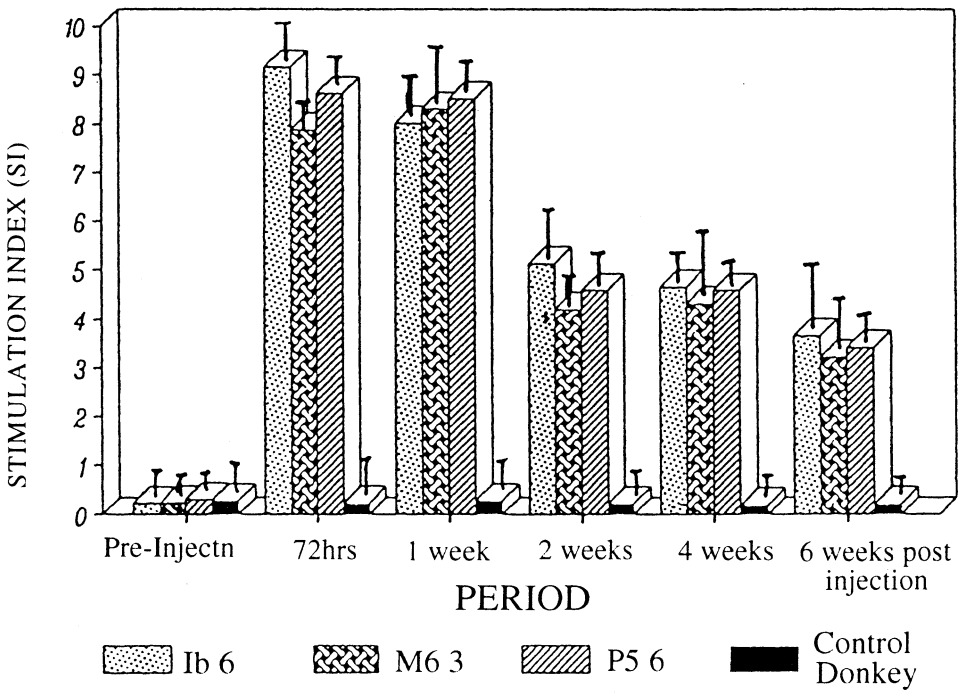

Fig. 2: PBL responsiveness to IV glycoprotein injection

Antibody titres to influenza virus

Haemagglutination inhibition and neutralizing antibody titres to influenza viruses are shown in Tables 1 and 2, respectively. The sera obtained at 7 and 21 days post challenge from the three test animals neutralized the allantoic fluid virus. The three animals did not show any overt clinical signs. The serum of unchallenged control animal failed to neutralize the allantoic fluid virus with extensive CPE observed at 48 h of incubation. Neutralization

titres at 7 days ranged from 16-640 in the three animals. At 21 days post challenge, titres ranged from 320-1024 in the two animals previously injected with virus glycoproteins while titres of 160-512 were observed in the previously uninjected donkey. The glycoproteins injections thus mimicked previous immunization of the injected animals. The demonstration of high neutralization titres in the two previously immunized animals probably indicate that memory B and T cells were activated in these animals.

Table 1
HI titers of influenza virus allantoic fluid antigens against post iv glycoprotein injection sera

Virus and Experimental animals	Positive Control to rabbit antiserum		Pre-injection serum	Post iv glycoprotein in injection serum test sera collection at						Negative control serum
	P56	F79		72 hrs	1 wk	2 wks	4 wks	6 wks	12 wks	
A/Eq/Ibadan/6/91 /Ib6/H3N8	<10	1280	<10	20	40	320	640	1280	1280	<10
A/Eq/Miami/63/M 63/H3N8 6 month old colt foal	<10	640	<10	10	20	160	160	640	1280	<10
A/Eq/Prague/56/P 56/H7N7 4 month old donkey foal	2560	10	<10	10	20	160	320	640	640	<10
A/Eq/Ibadan/4/91 /Ib4/H3N8 Control antigen	<10	1280	<10	10	20	320	160	1280	1280	<10

Table 2
Neutralization titers of influenza virus allantoic fluid antigens reacted with 2-fold serial dilutions of post iv glycoproteins injection sera

Virus and Experimental animals	Positive Control to rabbit antiserum		Pre-injection serum	Post iv glycoprotein in injection serum test sera collection at						Negative control serum
	P56	F79		72 hrs	1 wk	2 wks	4 wks	6 wks	12 wks	
A/Eq/Ibadan/6/91 /Ib6/H3N8	<1	160	<1	8	32	16	128	512	512	<1
A/Eq/Miami/63/M 63/H3N8 6 month old colt foal	<1	320	<1	8	16	16	64	512	512	<10
A/Eq/Prague/56/P 56/H7N7 4 month old donkey foal	320	1	<1	4	64	32	256	512	512	<10
A/Eq/Ibadan/4/91 /Ib4/H3N8 Control antigen	<1	160	<1	8	8	32	128	256	512	<10

In vivo Studies

The animals previously injected with virus glycoproteins and the unchallenged control donkey did not show any overt clinical signs of influenza infection except a slight increase

in rectal temperature in the test animals while the previously uninjected control adult female donkey had a temperature of 40 °C on day 5 post experimental infection followed by congestion of mucous membranes and bilateral oculo-nasal serous discharges on days 6 and 7. This animal was dull and anorectic and coughed occasionally from day 8 for about 3 days after which the signs abated. Paired sera collected on days 5 and 17 showed rising antibody titres of 20 and 160, respectively, in this animal while there was no significant differences in the titres of the 3 test animals. However, no virus was recovered from the nasopharyngeal washings after 3 passages.

Passage of PBL over nylon wool thrice depleted B cells presumably to <1% of total lymphocyte populations. Lymphocyte proliferation was induced in T-cell enriched cultures with SI of 5.8 ± 1.6 comparable to SI of 6.1 ± 0.2 in undepleted PBL cultures. This implies that equine influenza virus glycoproteins are both B and T cell mitogens.

Table 3
***In vitro* and *in vivo* stimulation indices of infectious virus, intact and denatured virus glycoproteins**

Stimulation agents	Stimulation index	
	In vitro	*In vivo*
Infectious virus	5.2 ± 1.3	4.7 + 0.6
Intact glycoprotein	6.4 ± 0.8	6.2 ± 1.7
Denatured glycoprotein	6.3 ± 1.1	6.8 ± 0.54

± = Standard Deviation

Concentration and isotypes of immunoglobulins produced

The concentration of Igs in tissue culture supernatants of stimulated PBL was 16.3 mg/ml. Fractionation of the Igs resulted in four protein peaks of IgGa, IgGb, IgGc and IgG(T) as shown in Fig. 3 while there was no discernible protein peak in the unstimulated cell culture supernatants.

Discussion

The above results demonstrate that the HA and NA glycoproteins of both equine-1 (H7N7) and equine-2 (H3N8) influenza A viruses are mitogenic for equine PBL *in vivo* as well as mitogenic and immunogenic *in vivo*. This *in vitro* mitogenicity is in accord with previous findings in human and murine models (Butchko et al. 1978; Armstrong et al. 1981; Anders et al. 1984; Hurwitz et al. 1985; Hackett et al. 1991) although these workers used normal and primed spleen cells while the *in vivo* mitogenicity and immunogenicity appear novel. The results also support the hypothesis that influenza virus glycoproteins are responsible for the mitogenic and immunogenic effects of influenza A viruses to induce lymphocyte proliferation.

While the *in vitro* lymphocyte responsiveness to both H3N8 and H7N7 equine influenza viruses had a similar pattern to some extent, the H7N7 virus gave better lymphocyte responsiveness than the H3N8 viruses. It is probable that the H7 HA is more mitogenic than the H3 HA. Armstrong et al. (1981) similarly found the human H2 HA to be more mitogenic than the H3 HA. The use of PBL from previously unexposed animal preludes initial priming of the lymphocyte which could imply that the lymphocyte responsiveness was a primary response unrelated to memory cells. It was thus possible to use this as a fruitful model for studying the overall immune responses of experimentally infected horses to

influenza virus infections *in vivo*. Our ability to record good lymphocyte proliferation in peripheral blood *in vivo* and to demonstrate HI, N and precipitating antibodies in the sera of test animals from 72 hours to 12 weeks post iv injection of virus glycoproteins lend support to and thus validate our hypothesis. The animals were also protected against experimental infection 6 months post glycoprotein injection while one donkey not previously injected with the glycoproteins developed overt clinical signs when challenged with an infectious virus. Influenza infection or immunization is usually followed by development of HI and N antibodies which neutralize the virus *in vivo* and inhibit virus release from infected cells as demonstrated with anti-HA antibodies (Dowdle et al. 1972). Anti-HA and anti-NA antibodies are therefore very important active factors in immunity to influenza virus infections. Murphy et al. (1982), Richards et al. (1992) and Guo et al. (1992) have equally reported high HI antibody titers in post influenza infection sera in man and equines. We have demonstrated similar findings in our present study and these observations lend credence to our results. It is thus probable that the neutralizing antibodies mediate immediate infection while memory cells are responsible for long term immunity. It is therefore reasonable to suggest that the antibodies we have demonstrated in this study could confer a long term protection in susceptible animals since the glycoproteins are capable of activating both humoral and cellular immune responses. The protection of our animals against infection at 6 months post glycoprotein injection supports this view. Hannant et al. (1988) similarly reported complete clinical protection for about 32 weeks in horses following a previous exposure of the animals to an H3N8 equine influenza virus.

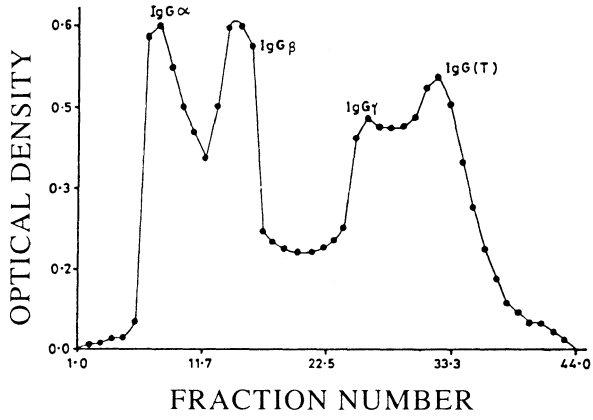

Fig. 3: Fractionation of immunoglobulins produced by proliferating PBL on DEAE-ion exchange chromatography.

Cellular immune response comprises a complex interaction between different cell types and molecules and induction of T cell response requires antigen processing, presentation and recognition by T cell receptors (Berzotsky et al. 1988). T cells recognize antigens/mitogens through their association with nascent MHC chains (Lanzavecchia 1990) and are thus activated. Although, it is difficult to relate the amount of injected mitogenic glycoproteins to the amount recognized by T cells in the peripheral blood of the test animals, it is quite possible to demonstrate the presentation to and the activation of T and B cells. We have quantitatively demonstrated and measured PBL responses as well as antibody responses to the mitogenic virus glycoprotein used in this study. Stimulation of B-depleted T lymphocytes by the virus glycoproteins clearly demonstrate that equine influenza virus glycoproteins are both B and T cell mitogens. This is in accord with previous observation by Armstrong et al. (1981) and Hurwitz et al. (1985) but contrasts with that of Anders et al. (1984) who reported that the HA glycoproteins of influenza A viruses were T cell independent B cell mitogens. Passage of PBL over nylon wool twice is expected to deplete B cell population to <1.4% (Anders et al. 1984). We passed PBL thrice over nylon wool to ensure complete removal

of B cells and we obtained similar SI for T-enriched cells and undepleted PBL cells thus leading us to conclude that the glycoproteins are B and T cell mitogens. Hurwitz et al. (1985) also demonstrated significant proliferation of purified T cell populations in response to isolated HA, NA, M and NP proteins of influenza A viruses. Our inability to further characterize the proliferating cells and determine the T cell subsets and lineages as well as the quantity of lymphokines produced by the activated T cells was due to lack of facilities and materials required to undertake such studies.

Induction of equine influenza virus glycoprotein mitogenicity and immunogenicity appears to be by direct binding of the virus protein antigens to receptors on the cell surface although activation of both classes I and II MHC restricted T cells has been reported (Hurwitz et al. 1985; Reay et al. 1989; Wysocka and Hackett 1990). This is reinforced by the inhibition of lymphocyte proliferation by MAbs and polyclonal antisera as demonstrated in the present study. Anders et al. (1984) also reported similar observation. Moreover, similar levels of lymphocyte stimulation were seen when infectious virus, intact and denatured glycoproteins were use as stimulants. It is thus reasonable to suggest that influenza virus-induced T cell responses do not appear to require the native conformation of the virus *per se*. The interaction between B and T cells and the resultant products are crucial to the animal's immune response. Cognate interaction involves conjugation of one B and one T cell (Sanders et al. 1988) and leads to activation of T cells which in turn can regulate cellular and humoral immune response either by further cell-cell contact or by release of lymphokines. The resting B cells can thus be stimulated to proliferate and produce antibodies whose isotype class switch from IgM to IgG, IgA and IgE could be mediated by T cell lymphokines. This is reflected in the fractionation of Igs produced by the proliferating cells into IgM, IgG and IgG(T) shown in Fig. 3.

Our results also demonstrate that previously immunized animals remain protected when challenged even after almost a year following previous immunization. This protection could be due to high titers of neutralizing antibodies in the serum and possibly mucosal immunity. However, higher titers were observed in the sera of previously immunized animals compared with the influenza-naive animal. This indicates that memory cells were induced with the resultant production of a large amount of Igs. The implication of this is that the protection engendered by these antibodies is related to activation of memory cells although mucosal immunity could have played some role in this. Our results also justify our conclusion on the possible use of influenza virus glycoproteins as denatured or proteolytic fragments in the study of a primary response of the horse to infection or even as immunogens for protecting valuable animals. However, it is pertinent to emphasize that the effects of carriers and adjuvants used in conventional vaccines should be taken into account since they can enhance or suppress the animal's immune response.

Most studies on the mitogenic effects of influenza A viruses on lymphocytes were carried out using tritiated thymidine incorporation into cellular DNA to assay cell proliferation. Such method is labour intensive, hazardous and expensive. The method of non-radioactive cell proliferation assay used in this study is more rapid, convenient, relatively cheaper, requires no radioactive incorporation and cell proliferation can be measured with an ELISA plate reader. The method is also less hazardous and the results obtained are comparable to those of radioactive incorporation assays or sophisticated particle counters. It is thus a method of choice in areas of the world where facilities are not readily available and where purchase and proper disposal of radioactive materials could be very difficult.

Buněčná imunitní identifikace virů A chřipky koní: studie imunogenity virů chřipky *in vitro* a *in vivo*

In vitro a *in vivo* studie imunogenity viru chřipky koní byly prováděny na koňském modelu za použití testu buněčné proliferace. Proliferující buňky byly charakterizovány a byla u nich stanovena indukce imunogenity. Virusy chřipky koní 1 (H7N7) a 2 (H3N8) byly mitogenní *in vitro*, avšak odpověď lymfocytů byla výraznější u prvního typu viru. Odpověď lymfocytů byla též pozorována *in vivo*, a to 72 h až 6 týdnů po intravenózní injekci virových glykoproteinů pokusným koním. Inhibice hemaglutinace a ochranných neutralizačních protilátek ukázala, že virusy chřipky koní a jejich povrchové glykoproteiny by mohly indukovat odpověď B a T lymfocytů u koní. Tato indukce nastala přímou vazbou virových povrchových antigenů na povrchové receptory, a to mimo již popsané aktivace T buněk s omezením histokompatibilního komplexu třídy I a II. Proliferující buňky produkovaly imunoglobulinové isotypy IgGa, IgGb, IgGc a IgG(T). V práci je diskutována role paměťových B a T buněk v ochraně imunizovaných zvířat.

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