CO-INFECTION BY SEMLIK FOREST VIRUS
AND MALARIAL PARASITE MODULATES VIRAL MULTIPLICATION,
PATHOGENESIS AND CYTOKINES IN MICE

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Summary:
Environmental, technological and societal factors continue to have a dramatic effect on infectious diseases worldwide and are considered to be facilitating the emergence of several infectious diseases at a time. Coinfection with different species of viral and malaria infections are currently emerging problems of dual infection in the developing as well as developed countries. Understanding of interactions between the host, malaria and virus infection is of current concern and we have initiated studies to delineate the mechanisms involved during the progression of Semliki forest virus (SFV) and Plasmodium yoelii (P. yoelii) infection in mice. Enhanced virus multiplication and up-regulation of cytokine mRNA level in P. yoelii and SFV coinfected mice were observed on day 4 post-infection compared to respective controls. Collectively, our observations indicate that malaria infection may influence virus multiplication, pathogenesis and upregulation of cytokine mRNA during co-infection in mice.

KEY WORDS: co-infection, virus, malarial parasite, P. yoelii, pathology, cytokine.

Environment contains a great variety of infectious microbes that may be potentially damaging and threaten our survival. Malaria continues to be one of the main public health problems in the world, especially in most of the African countries. Sharma, (1999) indicates that malaria is prevalent in northeastern and peninsular India. At present 90 countries or territories in the world are considered malarious; almost half of them being in Sub-Saharan Africa (WHO, 1993, 1996, 1996 and 1997).

Human populations are often infected with more than one infection at a time especially in developing countries (Cox, 2001; Howard et al., 2001). Co-infection with different species of malaria and a number of viral infections are frequent and this may impact an outcome and clinical manifestation. Non lethal strains of Plasmodium yoelii (P. yoelii) and P. chabaudi became lethal in mice infected with Rowson Parr virus (Cox et al., 1974). Similarly, Epstein Barr Virus, which normally causes mild infections, would contribute to the development of Burkitt’s lymphoma in individuals exposed to malaria (De Thé, 1985). Likewise, chronic infection with hepatitis B virus (HBV) may be a risk factor for severe malaria (Barcus et al., 2002). Malaria infection influence the course and pathogenesis of HBV infection in co-infected mice/humans (Pasquetto et al., 2000). Higher HIV-1 blood viral burden was recorded in patients with Plasmodium falciparum malaria (Hoffman et al., 1999). An association between HIV-1 infection and clinical malaria has been reported from Africa (Hoffman et al., 1999; Francesconi et al., 2001; Ter Kuile et al., 2004). P. falciparum infection modulates HIV-1 pathogenesis by activating lymphocytes and stimulating viral replication through production of cytokines (Xiao et al., 1998). Increased risk of malaria occurrence in HIV-infected pregnant women was reported in a cohort study (Ladner et al., 2002).

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Cohen (2003) has reported spread of malaria in HIV-infected pregnant women and found that co-infection with malaria significantly increased a mother’s risk of transmitting the AIDS virus to her baby before or during birth.

Co-infection with virus and malaria and other pathogens is an emerging problem. Understanding the various aspects of interactions between the host, virus and malarial parasite infection involving modulations in their infectivity, augmentation of the histopathology of infection and delineation of the mechanisms involved are the objective of the present investigation. A “Virus-malarial parasite mouse model” influencing dual infection with Semliki forest virus (SFV) and P. yoelii was used. SFV causes acute infection of central nervous system producing encephalitis. The virus replicates in different organs, tissues and within the CNS by infecting neurons (Smithburn & Haddow, 1944).

MATERIALS AND METHODS

ANIMALS, VIRUS AND CELLS

Swiss albino mice (10-12 gm. Body wt.) obtained from the Industrial Toxicology Research Centre breeding colony were maintained on a standard pellet diet and water ad libitum and were housed in environmentally controlled rooms (Temperature 25°C ± 2 and humidity 50 ± 5 %) under a 12 h dark light cycle. All procedures, maintenance and treatment of mice were in accordance with the guidelines for the care of animals by the Institutional Animal Ethics Committee.

VIRUS

Semliki forest virus (Smithburn and Haddow strain) obtained from American Type Culture Collection (ATCC), USA was passaged intracerebrally in Swiss mice (6-7 g). Mice showing moribund infectivity symptoms and typical hind limb paralysis were sacrificed and brain tissue was aseptically excised, homogenized and centrifuged at 5,000 rpm for 30 minutes. Supernatant serving as stock virus was stored at – 80°C.

CELL LINE

Baby hamster kidney (BHK-21) cell line obtained from National Centre for Cell Science (NCCS), Pune, was used for in vitro viral titration. Cells were grown in Minimum Essential Medium (MEM, Sigma, USA) supplemented with 10 % fetal bovine serum (FBS, Sigma, USA) and antibiotics (streptomycin 100 µg/ml, benzyle penicillin 100 units/ml and gentamicin 40 µg/ml) and maintained in MEM containing 2.5 % FBS and antibiotics.

VIRUS TITRATION

Brain tissue was homogenized (10 % w/v) in plain MEM, centrifuged at 5,000 rpm for 30 minutes, supernatant filtered by micro filter (Millipore, USA) and diluted. Several dilutions of virus samples were adsorbed on BHK-21 cell monolayer in 96 well microtitre plate, incubated for 48 hrs. at 37°C in humid atmosphere of 5 % CO₂ incubator (Heraeus Hera Cell model, Germany). The plates were later stained with 0.1 % crystal violet and examined under an inverted microscope for determination of virus titre by cytopathic effect assay as described earlier (Gupta et al., 2002). Cytopathic effect was graded on the standard scale of normal to virtually complete destruction of cell monolayer.

PARASITE

Plasmodium yoelii (P. yoelii) nigeriensis strain was used throughout the experiment. P. yoelii N-67 strain was maintained by serial blood passage as well as by transmission through mosquito vector, Anopheles stephensis.

PARASITAEMIA

Thin blood smears from P. yoelii infected mice were prepared taking a drop of tail blood. Fixed smears were stained with Giemsa stain and erythrocytes were observed for infected cells. Degree of parasitaemia was recorded as infected cells per 100 erythrocytes.

TREATMENT

Four groups of mice, ten in each group, were arranged and treated as control, given saline only; SFV control, infected subcutaneously with virus; P. yoelii control, infected intraperitoneally with malarial parasite and the experimental group, challenged with SFV and P. yoelii simultaneously. Three mice from each group were withdrawn and sacrificed on day 2 and day 4 post-infection for cytokine-mRNA analysis, virus titration in brain tissue and histopathological studies.

RIBONUCLEASE PROTECTION ASSAY OF CYTOKINES

We analyzed the expression of cytokine mRNA levels in brains of mice in all four groups using the Ribovuant Multiprobe Ribonuclease Protection Assay (RPA) kit (Phamingen, San Diego, CA, USA). Animals from each group were sacrificed on 4th day post-infection and brains were collected, snap-frozen, and stored at – 80°C. RNA was isolated from the frozen tissue samples using Trizol (Life Technologies Inc., USA), quantitated and equalized on formaldehyde agarose gels. For RPA, the protocols used were according to the manufacturer’s instructions. Briefly, 20 mg of each RNA sample was hybridized at 56°C for 12-14 h with a 32P-
UTP-labeled probe. The probe was prepared by transcribing the mouse cytokine mck-2b template set using T7 RNA polymerase. After hybridization, samples were subjected to Rnase digestion for 45 min. at 30°C. Ribonuclease-protected bands were then resolved on denaturing urea-PAGE gels, followed by autoradiography. The template used allowed us to study the differential regulations of IL-12p35, IL-12p40, IL-10, IL-1β, IL-1Ra, IGIF, IL-6, IFN-γ, macrophage inducing factor (MIF), L-32 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs served as housekeeping gene controls in the assay, to assure equal loading of RNAs.

**Histopathology**

To evaluate tissue damage, formalin fixed and paraffin embedded section of brain tissue were prepared from all four groups of mice, stained with hematoxylin and eosin (H & E) and examined microscopically for infection-induced lesions.

**Statistical Analysis**

The data was analyzed statistically by Student’s t-test for level of significance and presented as mean ± SE. Probability value (p value) was derived to represent the significant difference between the two groups.

**Results and Discussion**

Studies with SFV and *P. yoelii* showed an enhanced virus titre in the brain of mice challenged with SFV and infected with *P. yoelii* as compared to control on post infection (p < 0.001) (Fig. 1). Peritoneal blood parasitaemia was more elevated on day 4 in the group of mice co-infected with SFV and *P. yoelii* (p < 0.001) (Fig. 2). Cytokine profiles were studied using RPA kit. This showed that SFV infection led to up-regulation in mRNA levels of two cytokines. Expression of IL-1Ra and IGIF was detected in SFV + *P. yoelii* co-infected mice brain and was not evidenced in the *P. yoelii* group or the saline group (Fig. 3). A lower level of IGIF mRNA was observed in SFV infected mice brain. Histopathological analysis of the brain showed intact meningeal covering of cerebral cortex on day 4 after saline treatment (Fig. 4A). Meningeal oedema and necrotic debris in brain were present on day 4 following SFV infection (Fig. 4B). *P. yoelii* alone caused meningeal congestion (Fig. 4C). However, co-infection with SFV and *P. yoelii* caused marked meningeal congestion / greater meningitis and cortical degeneration (Fig. 4D). Overall the intensity of inflammation and cortical destruction was higher in the tissues of animals co-infected with SFV + *P. yoelii* when compared with single infection.

Cytokines are important mediators in inflammatory pathology of the central nervous system (CNS), including encephalitis and demyelinating diseases. Since IL-1Ra has been suggested to play a role in pathogenesis of viral infections, it is likely that *P. yoelii* accelerates viral multiplication through its up-regulation. Up-regulation in mRNA level of cytokine IL-1Ra in brain of mice co-infected with SFV and *P. yoelii* support above
contention. Recently, Haque et al. (2004) have demonstrated up-regulation of cytokine mRNA expression in the spleen and lungs of Balb/c mice infected with P. yoelii and MHV. Co-infection of the host may have different consequences for each of the infectious agents. In mixed infections, the burden of one or both the infectious agents may be increased, one or both may be suppressed or one may be increased and the other suppressed. Immunosuppression in mice and humans was reported to be characteristically associated during malaria infection. Enhancement of virus titre in co-infected animals is explainable in these terms. Freitag et al. (2001) have shown that malaria infection induces virus expression in human immunodeficiency virus transgenic mice by CD4T cell-dependent immunoactivation. Pasquetto et al. (2000) reported host virus interactions during malaria infection in hepatitis B virus. Wabwire et al. (2001) have shown the effect of HIV-1 infection and malaria parasitemia. All these studies support our contention that co-infection with malaria or other parasites may enhance viral multiplication. Although the observations are of preliminary nature, however, the implications of the present investigation are indicative of far reaching effects and adds new dimension to the problem of predisposition to viral and malaria co-infection and in-depth studies are required to understand host-virus and malarial parasite interaction since multiple infections may have important implications for designing control programmes against parasitic diseases.

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