correction is not possible (as in vasal-aplasia). However, in most of these men, a good number of spermatozoa with variable motility could be obtained by epididymal aspiration. It is not always possible to use the spermatozoa obtained by epididymal aspiration for Assisted Reproductive Technology as this requires an additional expense and simultaneous preparation of the wife. Therefore it was decided to try and cryopreserve epididymal spermatozoa obtained from caput epididymis (in men undergoing scrotal exploration) so that this could be used at a later date if necessary.

The subjects for the study were three men undergoing scrotal exploration for obstructive azoospermia in 1991. Informed consent was obtained from all these men. Epididymal spermatozoa were aspirated from caput epididymis into 1 ml of Earle's balanced salt solution. The prefreeze spermiogram was done. This was then mixed with an equal volume of Matheson Carlborg–Gemzell medium (cryoprotectant[4]) and cryopreserved in a biological freezer at $-70°C$.

Good motile spermatozoa were obtained from all the three men from caput epididymis. The sperm concentration prefreeze were twelve million, ten million and thirty-five million per ml with 30%, 40% and 60% progressively motile spermatozoa. Vaso epididymal anastomosis was done on two men. One man had vasal aplasia. One of these men, two months later, showed a sperm concentration of eighty million per ml with 30% motility and his wife was pregnant a month later. She has since delivered a baby. The other man had no spermatozoa at the first follow up, a month later, and has not reported back since then. As these three men were not keen on Intra Uterine Artificial Insemination Husband or ART for their wives, the spermatozoa so obtained from the caput were frozen with their consent. After one month the sample was taken out and thawed and it was found that the spermatozoa in all the three samples had still maintained 40–50% of their prefreeze motility.

Surgical correction of obstructive azoospermia generally yields poor results (20%). Unless and until experience is gained with single tubular anastomosis, the results of vaso epididymal anastomosis are not likely to improve. However, many centres have good experience with GIFT/IVF by conventional means or by Micro Insemination and Sperm Transfer (MIST), Sub-Zonal Insemination (SUZI) or Intra Cytoplasmic Sperm Injection (ICSI). For all these techniques, the number of spermatozoa required is far less than for *in vitro* fertilization. Therefore, while attempting vaso epididymal anastomosis for obstructive azoospermia, if the couple are prepared, one could also undertake IUAIH or ART. If, however, the couple are not prepared, one could attempt to cryopreserve the epididymal spermatozoa and use the cryopreserved spermatozoa for ART later if the surgical correction were to be unsuccessful.

All mammalian cells are not cryopreservable. It is not known what offers the spermatozoa the cryopreservable capacity. The fact that epididymal spermatozoa could be cryopreserved indicates that human spermatozoa do not require the presence of seminal constituents for being cryopreserved.

Epididymal spermatozoa which are mature, motile and capable of fertilization are also cryopreservable. It is suggested that all attempts at surgical correction of obstructive azoospermia should be accompanied by an attempt at IUAIH/ART or at least cryopreservation of epididymal spermatozoa so obtained.

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Cytokine antagonism by active vaccination

The soluble mediators, known as cytokines, regulate immune response as well as effector phase of immune reactions. They are characterized by functional pleiotropy and redundancy and act as a 'network'. They are required for a number of normal reactions in the body but produce pathological changes when they are present at the wrong place and in wrong concentrations. Their role in a number of diseases has generated keen interest in the cytokine antagonists and their potential therapeutic use. The cytokine antagonists are nonpeptide molecules, soluble receptors, IL-1ra/ mutated cytokines and autoantibodies[1]. Besides these, the adverse effects of cytokines have been neutralized successfully by passive immunization of animals by anticytokine-antibodies[2–6]. On the other hand, the non-neutralizing type of antibodies contribute to a positive therapeutic response by better targeting of the cytokines to the appropriate cells[7,8]. We have successfully used a novel approach to actively immunize mice with a dengue virus-induced cytokine, the mouse Cytotoxic Factor (mCF), which acts as 'pathogenesis-related protein', to protect against the pathological effects[9]. We wish to draw attention to this potentially therapeutic approach of 'anti-disease vaccination'.

During dengue type 2 virus (DV) infection of mice, a unique cytokine mCF is produced by T lymphocytes. mCF is a 43 kD molecule on native PAGE and has an isoelectric point of pH 6.5. The sequence of 19 amino acids of the *N*-terminus of mCF (compared at Gen Data Base, Distributed Information Centre, Indian Institute of Science, Bangalore) differs from those of other cytokines and DV-specific proteins[10]. Northern blot tests done with the oligonucleotide probes derived from this sequence shows the presence of mRNA for mCF in the spleen cells of DV-infected mice. The cDNA library of mCF has been constructed in λgt 11 and expressed in *Escherichia coli*

(Chaturvedi *et al.*, unpublished data). mCF kills mainly H-2A negative macrophages and T helper cells etc. of a variety of animal species by inducing Ca^{2+} influx[11–14] and nitrite production (A. Misra, R. Mukerjee and U. C. Chaturvedi – communicated). mCF is capable of reproducing various pathological lesions that are seen in human cases of dengue haemorrhagic fever/shock syndrome (DHF/DSS) which include increased vascular permeability and damage to the blood–brain barrier[4,6] (Figure 1). Treatment of human blood leucocytes with mCF *in vitro* produces changes[15] that are described in cases of DHF[16].

Recently, presence of an mCF-like cytokine has been shown in the sera of the cases of DHF/DSS (ref. 17). This human CF (hCF) has been purified by chromatography and characterized (Chaturvedi *et al.* – unpublished data). Further, production of hCF by human peripheral blood CD4 positive T cells has been shown by *in vitro* stimulation by DV (ref. 18). T cell-enriched subpopulation could be stimulated directly to produce hCF but the yeild of hCF was enhanced in the presence of monocytes/macrophages (Mϕ). This shows that the effect of DV can be both direct or via monocytes. The Northern blot analysis using oligonucleotide probe prepared on the basis of amino-terminal sequence of mCF shows the presence of mRNA in T cells[18]. A number of characteristics of mCF are shared by hCF (Table 1), including molecular weight, reaction in Western blots and capacity to increase capillary permeability[19–22] (R Mukerjee and U. C. Chaturvedi – communicated). This is an extremely important breakthrough in DHF research as mCF has been shown to be a 'pathogenesis-related protein' in mice.

Mice immunized with 5 µg of mCF mixed with Freund's incomplete adjuvant were challenged with 3 µg mCF at weekly intervals up to 48 weeks. Protection against the mCF-induced increase in capillary permeability and damage to the blood–brain barrier was studied in normal control and immunized mice. A complete protection is observed at the 4th week which persisted at higher levels up to the 16th week and then declined sharply. A breakthrough in the protection occurred with higher doses of mCF in a dose-dependent manner. mCF-specific antibodies are present in the sera obtained from the immunized mice as detected by ELISA and Western blot test and by neutralization of the cytotoxic activity of CF *in vitro*. The peak antibody titres correlated with peak protection. Intracerebral challenge with a lethal dose of DV prolongs mean survival time and delays onset of symptoms of sickness in immunized mice compared with normal mice, but the titre of the virus in the brain was similar[9]. Recently, the effect of various adjuvants, including Freund's incomplete adjuvant, alhydrogel, bacille Calmette-Gucrin (BCG), normuramyl-L-*N*-methylalanyl-D-isoglutamine octalymide (compound No. 84/246, synthesized at the Central Drug Research Institute, Lucknow and kindly provided by K. B. Mathur) and tetanus toxoid (TT) on the immunogenicity of mCF and their optimum doses have been studied. The best effect is obtained with 84/246 followed by that with TT (ref. 20).

Paralysis and death of mice occur due to replication of DV in the neuron cells, while damage to the blood–brain barrier resulting in cerebral oedema and associated symptoms is caused by both virus and mCF. On the other hand, cerebral oedema is produced, but not the mortality, by mCF on intraperitoneal (i.p.) inoculation of mice with DV, as the virus does not enter the brain[6]. By mCF-immunization, neither the virus titre nor the DV-induced paralysis and death could be prevented, but the latter are significantly delayed by 3–9 days and the mCF-induced pathological lesions are prevented[9,20]. The immunization provides almost 90% protection against damage to the blood–brain barrier on challenging with DV i p. Therefore, this approach may be very useful in cases where the virus does not enter the central nervous system (CNS). For example, in the cases of human dengue, signs and symptoms of cerebral oedema and rarely of encephalitis may be observed but the virus has never been detected in their CNS[6]. In spite of extensive work done to understand the pathophysiology and pathogenesis of DHF/DSS, the precise mechanism remains unclear[23], necessitating an indepth understanding of the pathophysiology of dengue syndromes to possibly open up new strategies to develop efficient vaccines.

Dengue illness is one of the most rapidly expanding diseases of the tropics, with over two billion people at risk of infection and millions of cases occurring every year[24]. The epidemics of dengue are widespread in this country and repeatedly occur year after year[25]. Dengue viruses are of four closely related types and yet each is sufficiently distinguishable from the others, such that sequential infection may lead to life-threatening haemorrhages and shock reactions. This has caused several problems in the development of a vaccine with the result that no vaccine against dengue is yet available for general use The ultimate

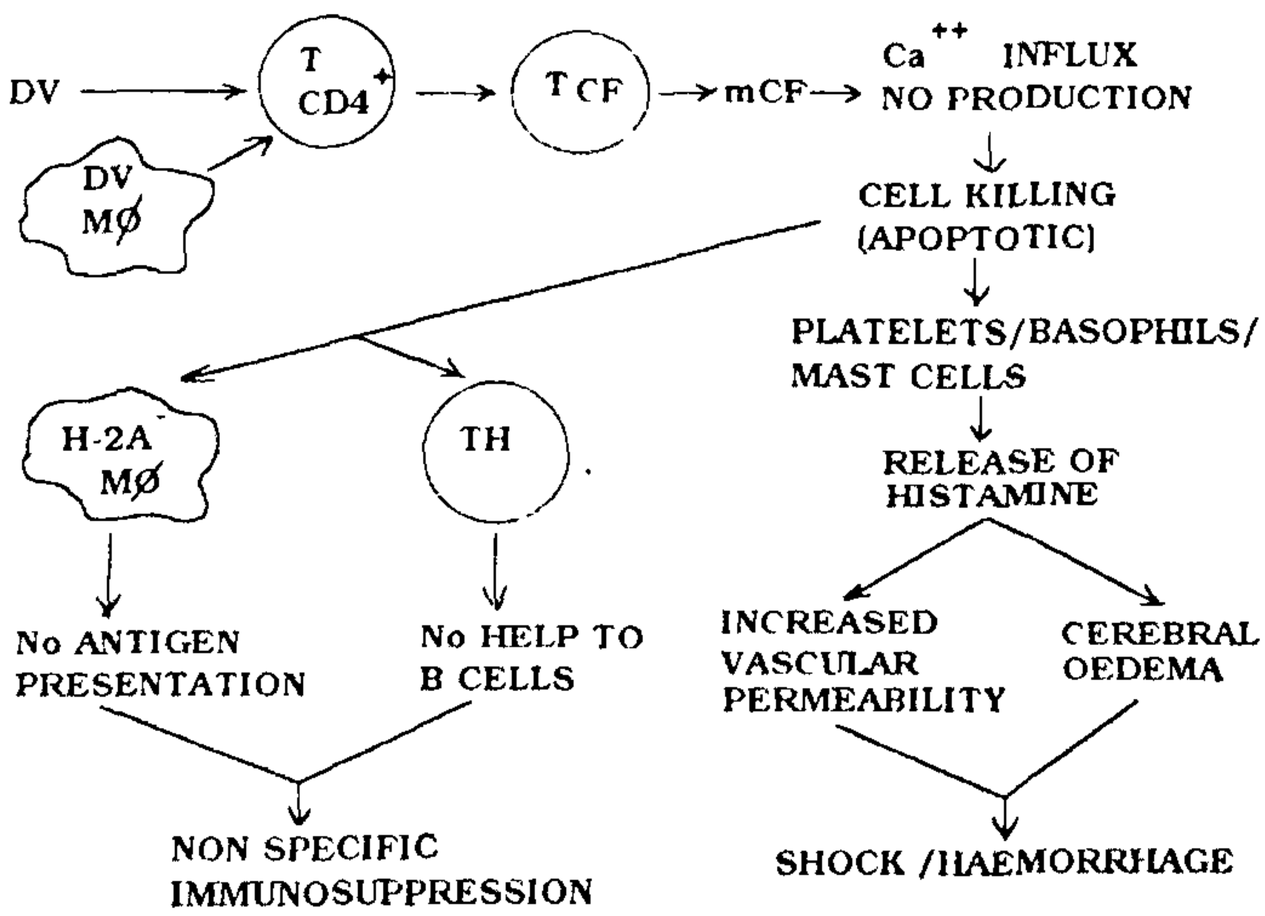

Figure 1. Production and mechanisms of action of mouse cytotoxic factor[19].

Table 1. Comparison of mouse (mCF) and human (hCF) cytotoxic factor[19]

Property	mCF	hCF
Inducer	DV[21]	DV[18]
Producer cell	L3T4 + T cells[21]	CD4 + T cells[18]
Target cell for assay	Mouse spleen cells[22]	Mouse spleen cells[18]
Mol wt on PAGE	43 kD (ref. 10)	43 kD (ref. 19)
Isoelectric point	6 5 (ref 10)	4 5–5.2 (ref 19)
N-Terminal sequence	19 amino acids known[4]	*N*-terminal blocked*
Effect on capillary permeability	Increased[4]	Increased[†]
Reaction with anti-mCF-antisera		
– Cytotoxicity	Neutralized[4]	Neutralized*
– ELISA	Positive[17]	Positive[17]
– Western blot	Positive[10]	Positive[18]
– Dot blot	Positive*	Positive[18]
– mCF/hCF-induced increase of capillary permeability	Inhibited[4]	Inhibited[†]
Reaction with anti-hCF-antisera·		
– Cytotoxicity	Neutralized*	Neutralized*
– ELISA	Positive*	Positive*
– Western blot	Positive*	Positive*
– mCF/hCF-induced increase in capillary permeability	Inhibited[†]	Inhibited[†]
Northern blot[‡]	mRNA present in T cells*	mRNA present in T cells[18]

*Personal unpublished data
[†]Mukerjee, R. and Chaturvedi, U. C. – communicated.
[‡]With the oligonucleotide probe prepared on the basis of amino-terminal sequence of mCF.

aim for dengue vaccine development should be the prevention and control of haemorrhages and shock which are life-threatening rather than the milder form of benign dengue fever. The data obtained so far indicate feasibility of developing an 'anti-disease vaccine'[9,20] for prophylaxis against DHF/DSS. On analysis of amino-terminal sequence of mCF[10] (at Gen Data Base, Bangalore) a projection of three antigenic sites was obtained on the molecule. Recombinant CF (rCF) has been obtained by expression of λgt 11 packed with cDNA in *E. coli* and some of its properties have been compared which show similarity between the native mCF and rCF (U. C. Chaturvedi – unpublished data). A question that needs to be addressed is whether such a vaccine could ever find practical use because it is based on immunization against a self-protein. Presence of autoantibodies to a number of cytokines have been described and their role were discussed[1]. Anticytokine autoantibodies are known to block the activity of the cytokines and are found in the serum for interleukin-1a (IL-1a), IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α and IFN-α. Small complexes between such antibodies and cytokines do not activate complement if IgG4 antibodies are involved, and do not precipitate *in vivo*, but are active as inflammatory complexes, therefore, their therapeutic use should be considered carefully[1]. Bendtzen *et al.*[7] have proposed that a major role of anticytokines autoantibodies is to facilitate, rather than neutralize, functions of cytokines in the body by acting as specific physiological carriers and regulators of cytokines. For effective immunization it is essential that such epitopes on cytokines are used which generate neutralizing antibody only. This approach has the advantages and disadvantages of an active vaccination, but vaccine strategies directed at the primary cause (the cytokine) rather than infective agents need serious consideration, more so when effective vaccines are not available for several agents.

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