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Evaluation of the cellular immune responses on patients with febrile illnesses during a Dengue epidemic in the State of Minas Gerais, Brazil: Preliminary results: In the present study, we evaluated by flow cytometry the activation of both $CD4^+$ and $CD8^+$ T cells through the analysis of $CD3^+$ cells co-expressing either $CD4^+$ $CD69^+$ or $CD8^+$ $CD69^+$ in patients with primary Dengue infection. We also evaluated the production of cytokines using flow cytometry and intracellular detection of IFN-γ, IL-4, TNF-α, and IL-1β. Forty patients enrolled at outpatient clinic with febrile illnesses were screened using an fast immunochromatographic test for the detection of anti-Dengue specific IgM and IgG. Seventy-six percent of patients with fever were positive for Dengue. PBMC were isolated and both activation status and cytokine production were evaluated. From day 0 to day 7 the co-expression of CD4 and CD69 molecules on patients positive for Dengue virus. The activation of CD8 T cells from Dengue patients increased significantly when compared to non-Dengue patients and healthy controls. The analysis of cells producing cytokines showed a predominance of cells producing TH1 cytokines (IFN-γ) at day 0 and 7. It also revealed an up-regulation on the production of TNF-α and IL-1β by monocytes, interestingly these two cytokines are associated with febrile manifestations and cells producing them were high at days 0 and 7 but low at day 3. Although preliminary, the results presented here suggest a central role for $CD8^+$ T cells and for Th1 cytokines in primary infections with Dengue virus.

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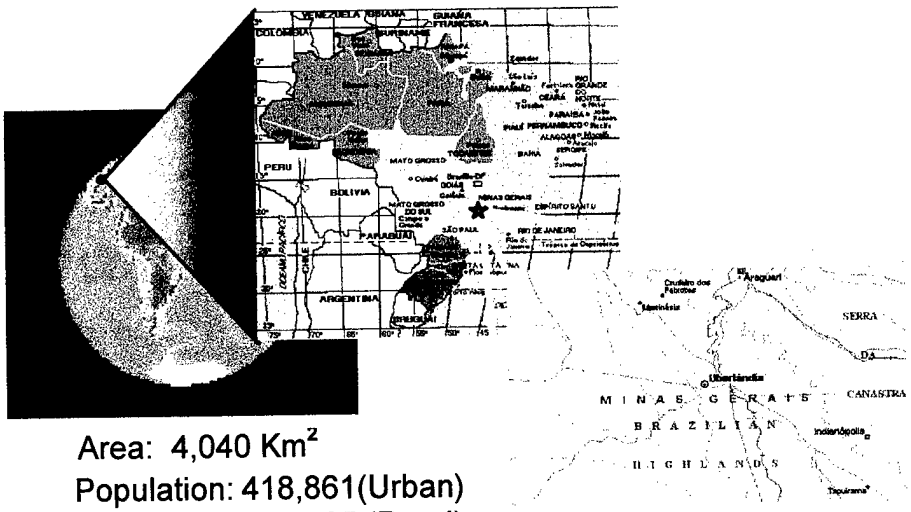
PROGRESS REPORT ON THE PRIMARY DENGUE CYTOKINE PROFILE PROJECT UFES/CBM/NDI & WRAIR/USAMRU-B, 1999

This study was conducted with 40 febrile patients from Uberlandia, MG during an outbreak of Dengue Fever (Figure 1).

Figure 1 - Uberlândia, Minas Gerais State, BRAZIL



Area: 4,040 Km^2
Population: 418,861(Urban)
8,735 (Rural)

We evaluated by flow cytometry the activation of both $CD4^+$ and $CD8^+$ T cells through the analysis of $CD3^+$ cells co-expressing either $CD4^+$ $CD69^+$ or $CD8^+$ $CD69^+$ on patients with primary Dengue infection. Intracellular IFN-γ, IL-4, TNF-α, and IL-1β were evaluated using specific monoclonal antibodies and flow cytometry. Blood samples were taken from 40 patients suspected of having primary Dengue at days 0, 3, 7 and 60 after enrollment in the protocol. PBMC were separated and both the activation status and cytokine production were studied.

Twenty-nine out of the 40 febrile patients enrolled in the study were positive for Dengue, the diagnosis was performed using an immunochromatography test for the detection of IgM and IgG specific for Dengue viruses (Dengue Fever Test, PanBio, Windsor, Australia) (Figures 2 & 3). The studied population consisted of 18 males (12-63 years, mean average $\pm$ STD = 32.4 $\pm$ 13.9 years) and 22 females (16-43 years, mean average $\pm$ STD = 27.6 $\pm$ 9.6 years). The frequency of the most frequent symptoms observed within the studied population is listed on Figure 4.

Figure 2 – Results from Dengue Rapid Test – PanBIO.

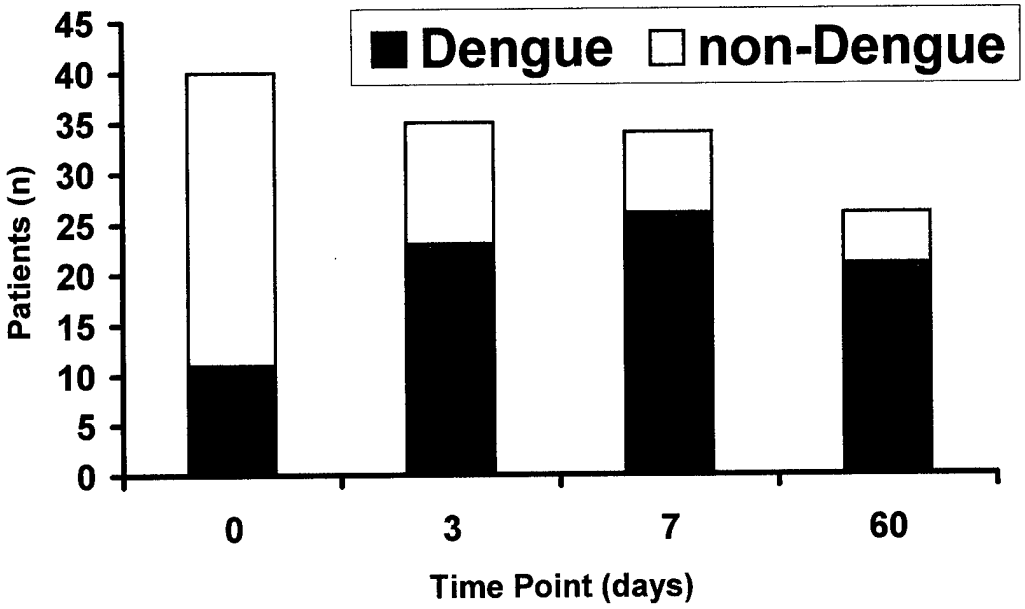


Figure 3 – Isotypes from Dengue specific antibodies at days 0, 3, 7, and 60.

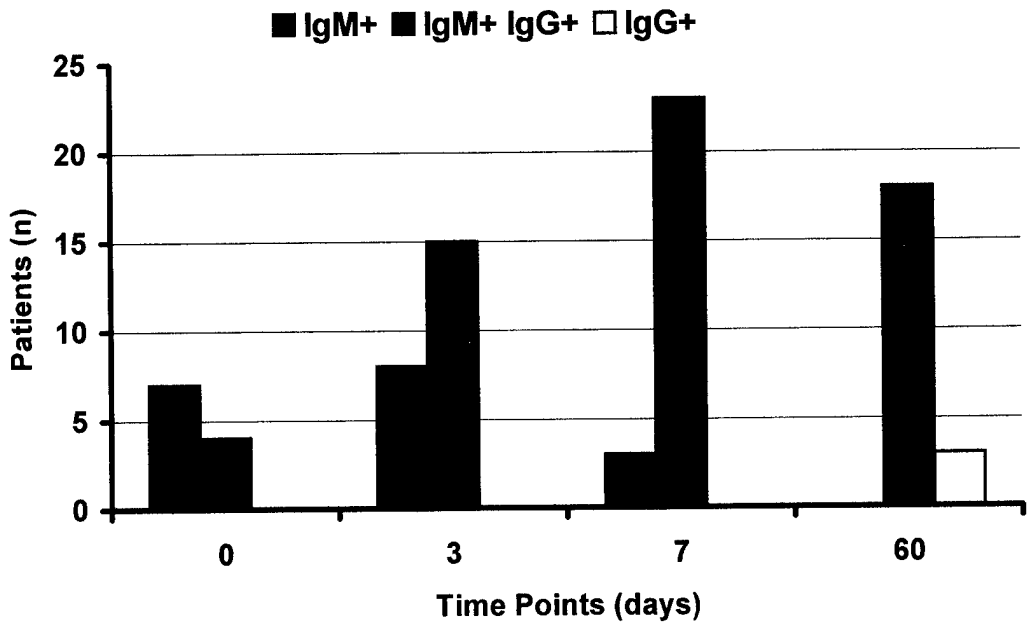
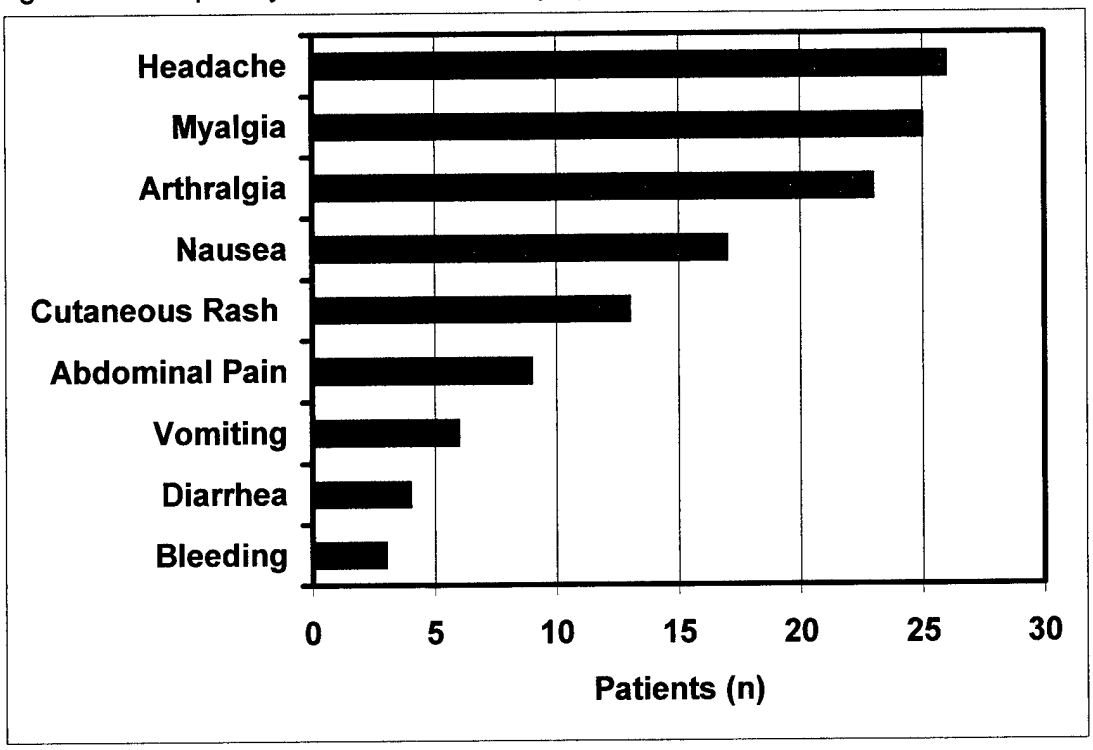


Figure 4 – Frequency of most common symptoms among Dengue^{+} patients.



The frequency of CD3^{+} lymphocytes on both Dengue- and non-Dengue-positive patients is presented on figure 5a and 5b.

Figure 5A – Number of lymphocytes on the studied population.

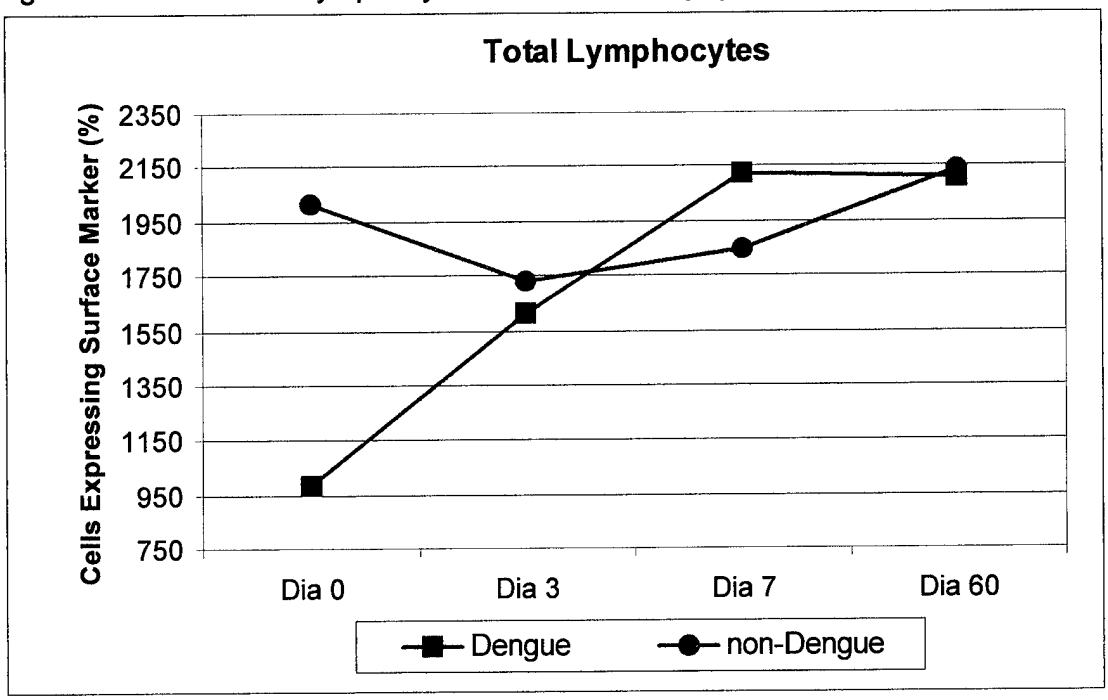
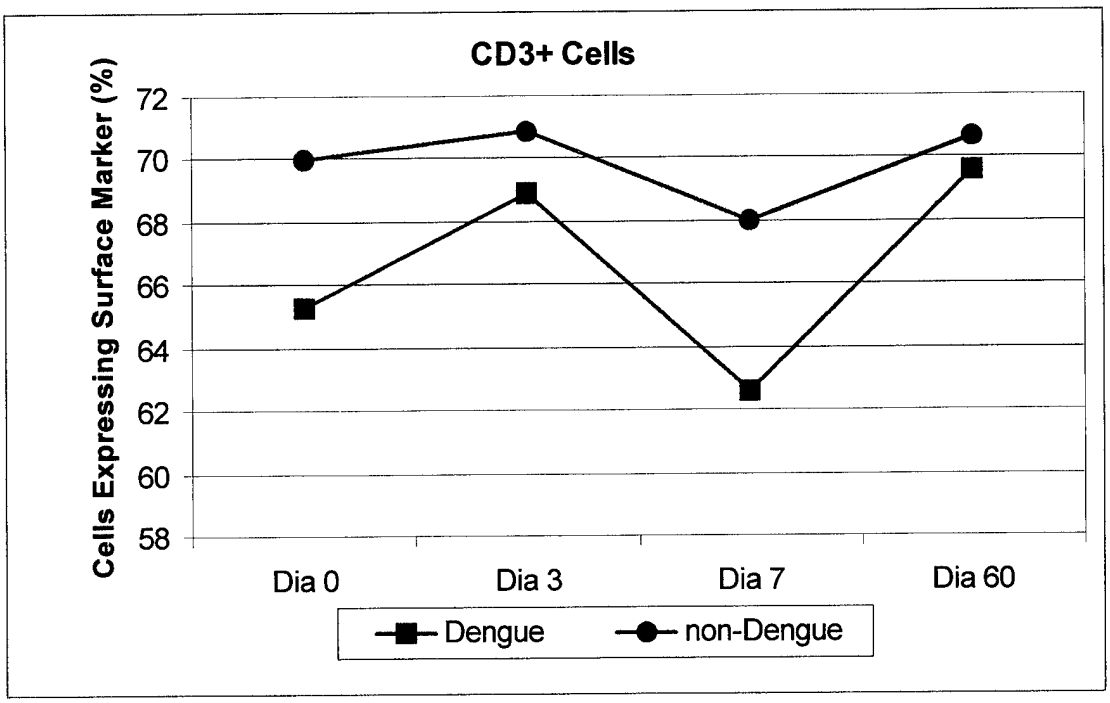
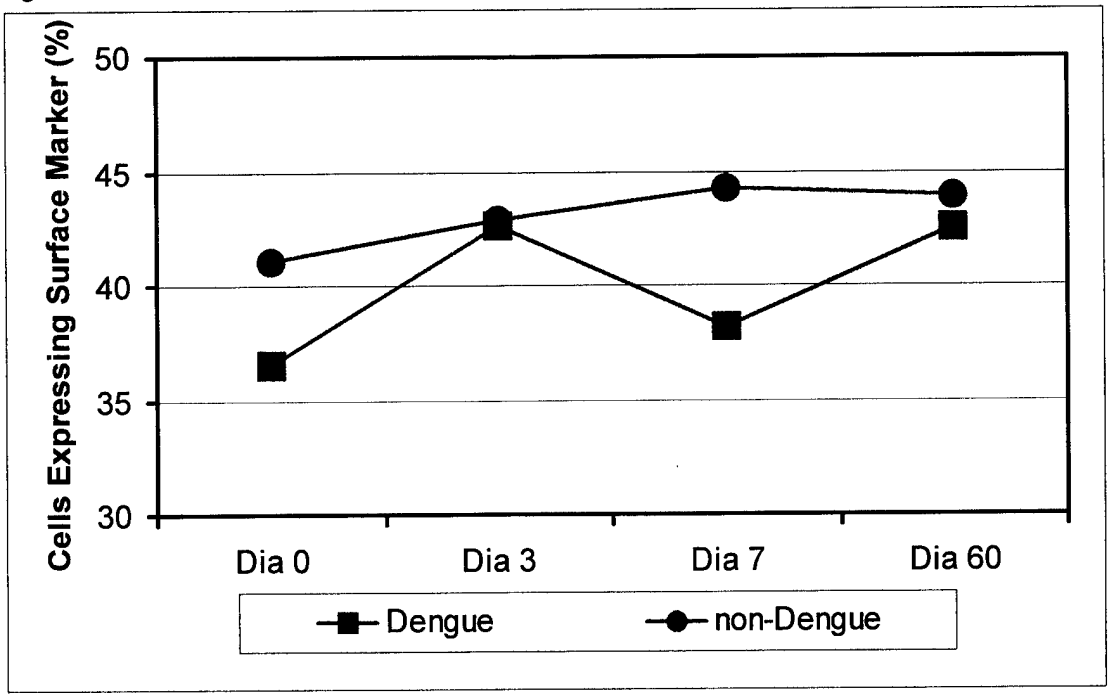


Figure 5B – Number of $CD3^+$ lymphocytes on the studied population.



As expected, lymphopenia was present on days 0, 3, and 7 only among $Dengue^+$ patients, especially on the $CD3^+CD4^+$ cell population (Figure 6).

Figure 6 – Levels of helper T cells on both $Dengue^+$ and non-$Dengue^+$ patients.



Conversely, $CD3^+CD8^+$ population was elevated from days 0 to 60 on the $Dengue^+$ group (Figure7). Activated $CD3^+$, T cells co-expressing CD69, were more prevalent on $Dengue^+$ patients at days 0 and 3, but at the same level of non-$Dengue^+$ patients on days 7 and 60 (Figure 8). Although, $CD4^+$ T cells were present at a lower frequency on $Dengue^+$ patients, $CD4^+$ co-expressing $CD69^+$ were elevated among these patients from days 0 to 7 (Figure 9). The same was observed for activated CD8 T cells (Figure 10).

Figure 7 – Frequencies of $CD3^+$ $CD8^+$ cells.

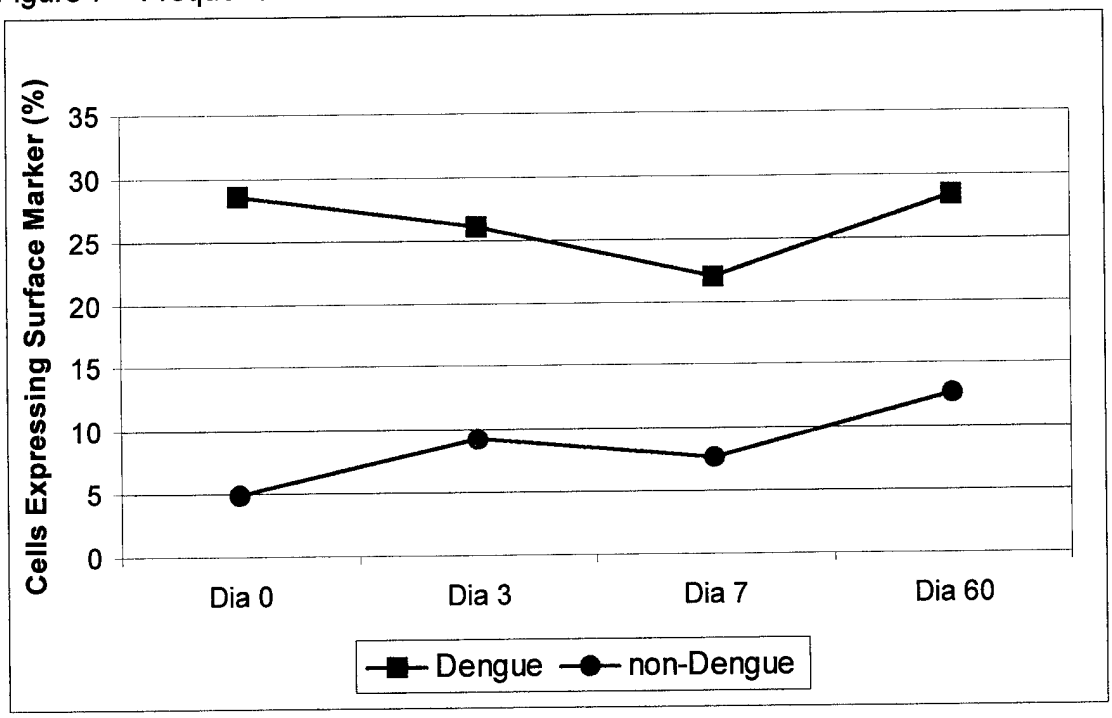


Figure 8 - Frequencies of activated $CD3^+$ T cells

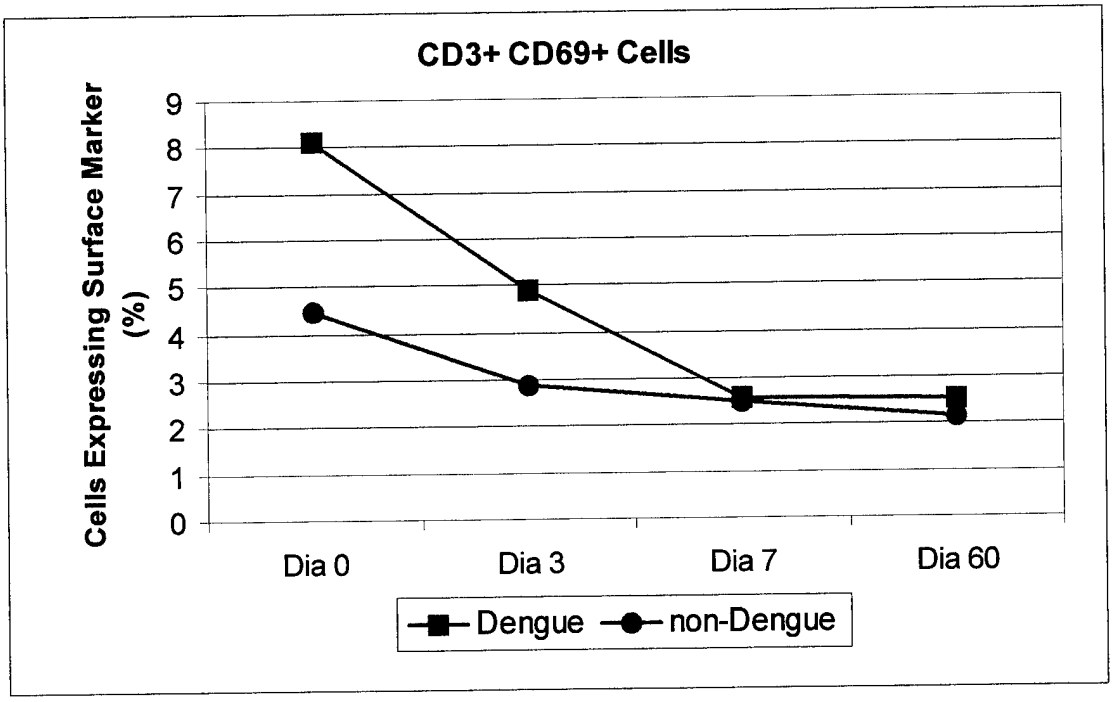


Figure 9 – Activated $CD4^+$ T cells on both $Dengue^+$ and non-$Dengue^+$ patients.

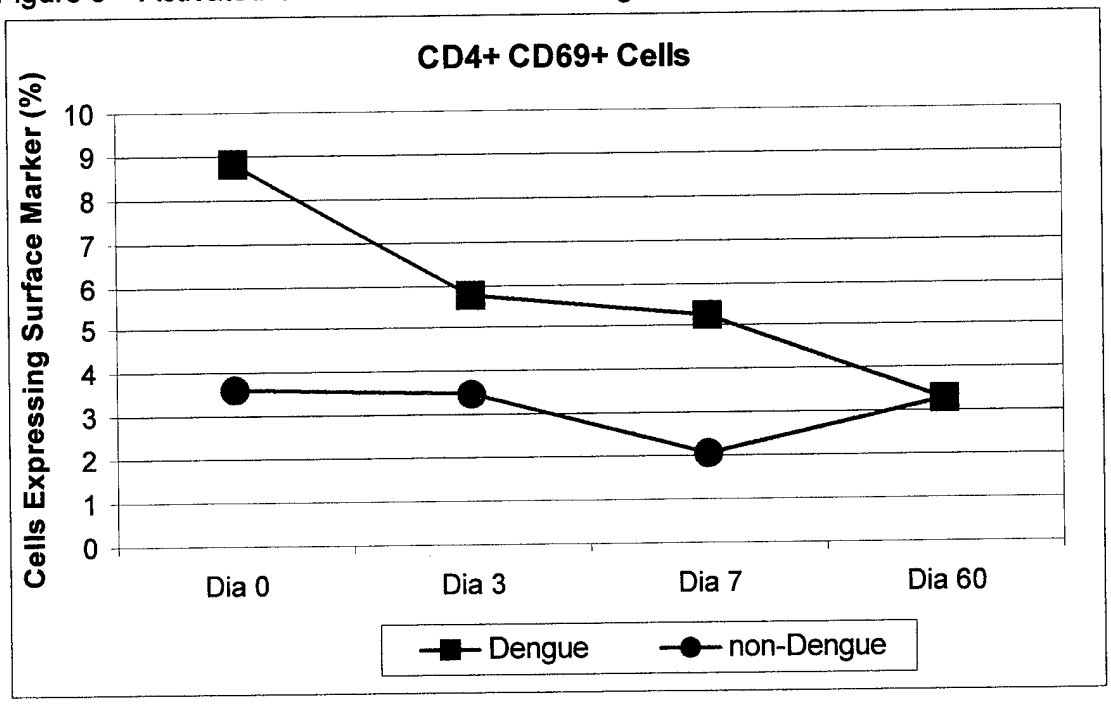
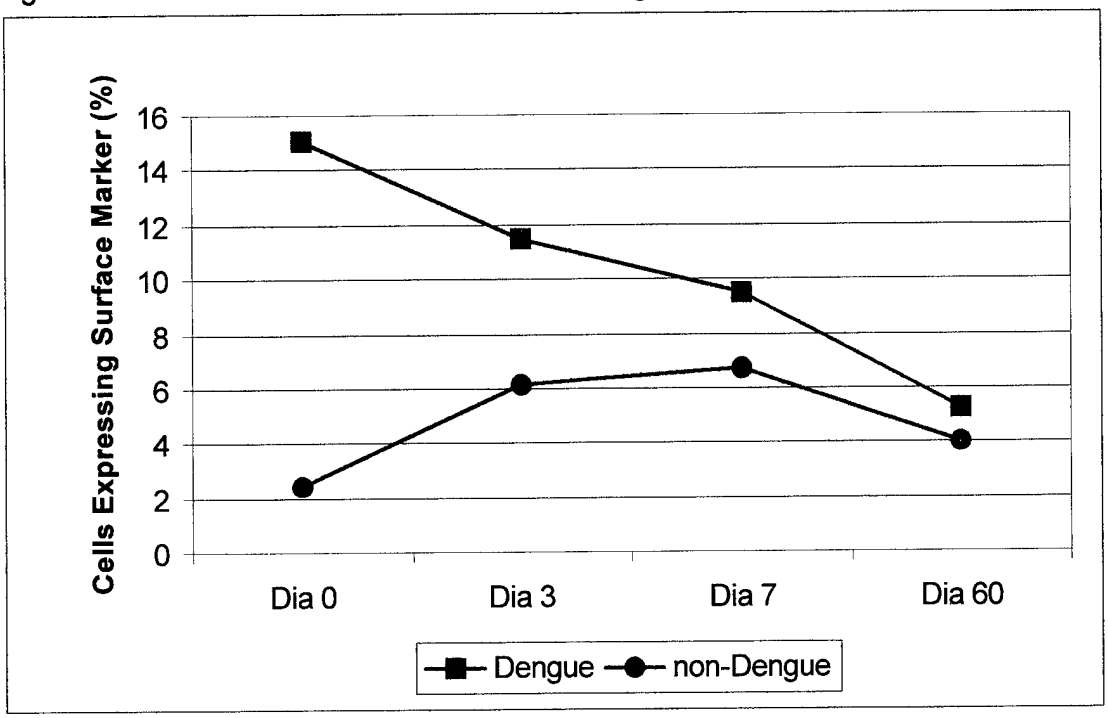


Figure 10 - Activated $CD8^+$ T cells on both $Dengue^+$ and non-$Dengue^+$ patients.



Although the levels of natural killer cells were lower on $Dengue^+$ patients, we observed that at days 0 and 7 activated NK cells were higher among $Dengue^+$ patients than on the non-$Dengue^+$ group (Figure 11 and 12).

Figure 11 – Levels of natural killer cells.

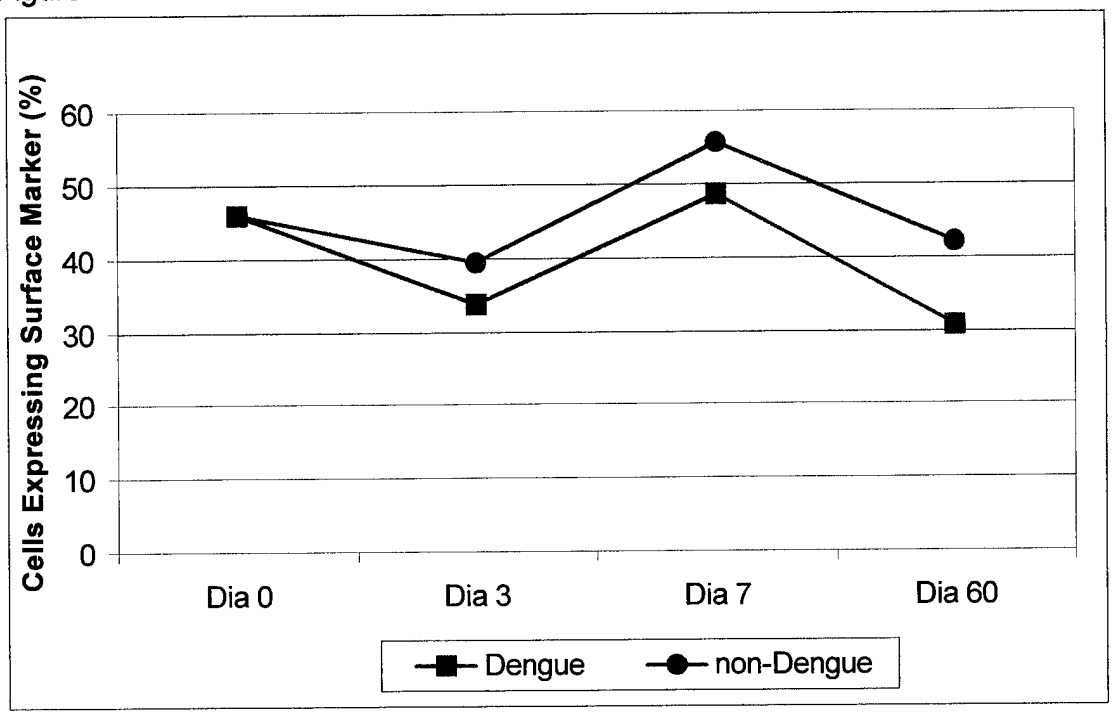
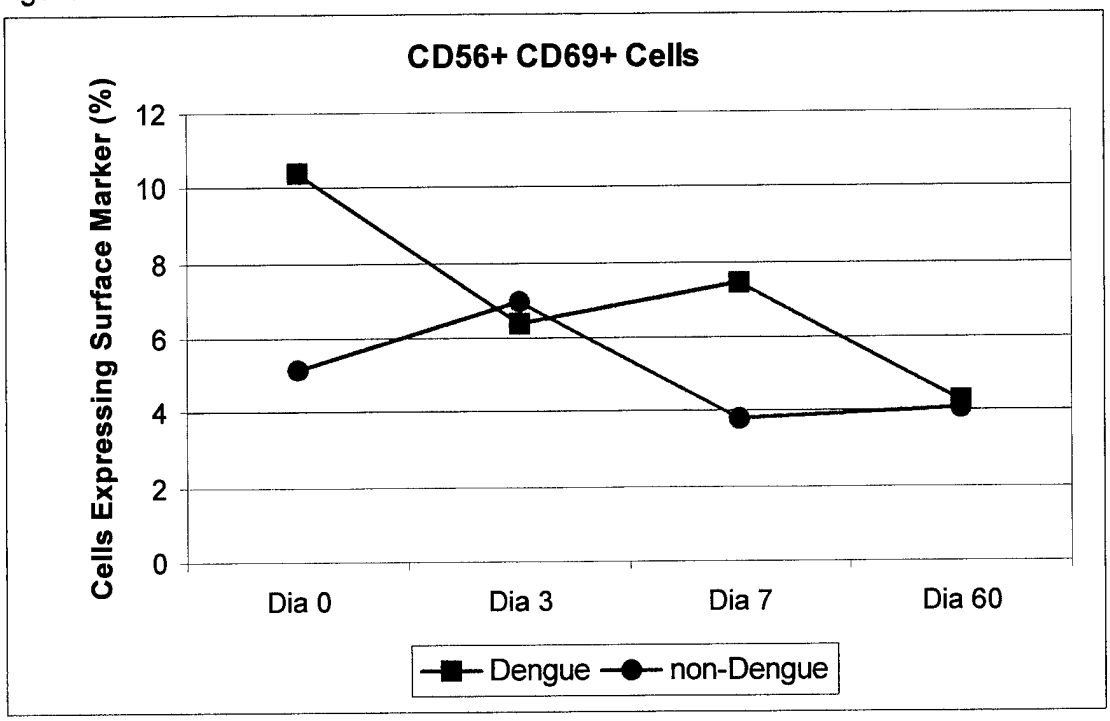


Figure 12 – Activated NK cells.



The analysis of cells producing cytokines showed a predominance of cells producing TH1 cytokines (IFN-γ) 2.5% at day 0 by $CD4^+$ T cells (Figure 13). It also revealed an up-regulation on the production of TNF-α and IL-1β by monocytes (Figures 14 & 15).

Figure 13 – $CD4^+$ T cells producing IFN-γ.

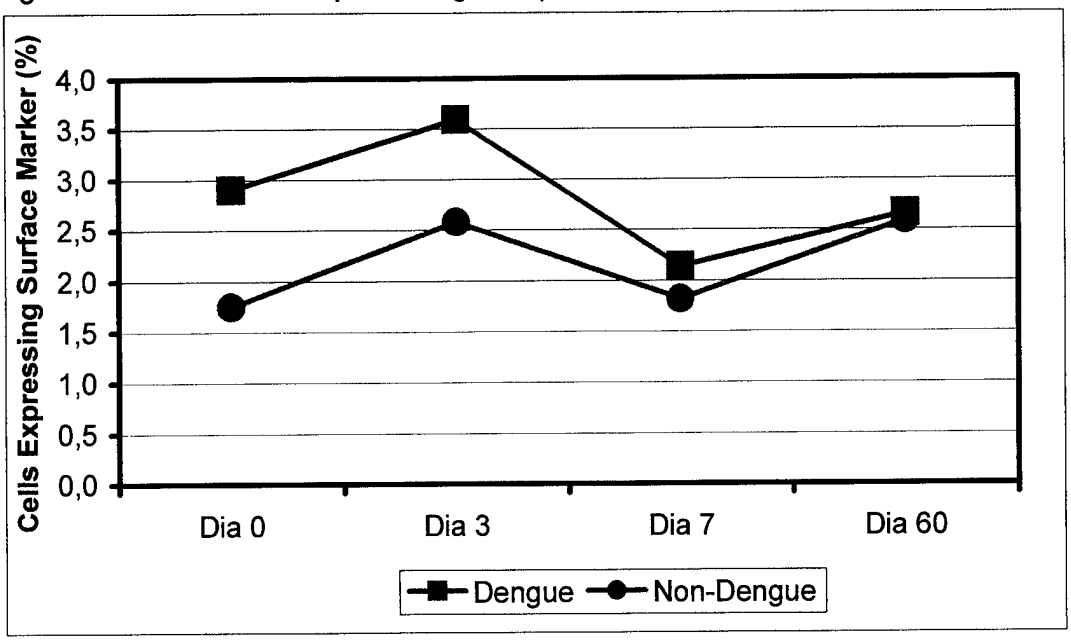


Figure 14 – Production of TNF-α by monocytes.

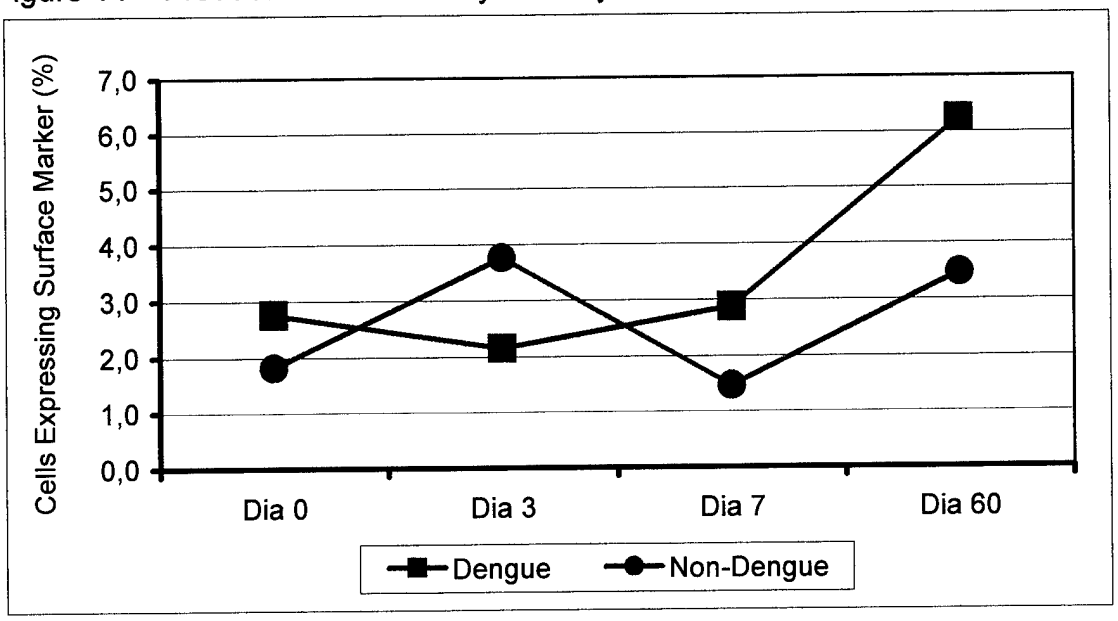
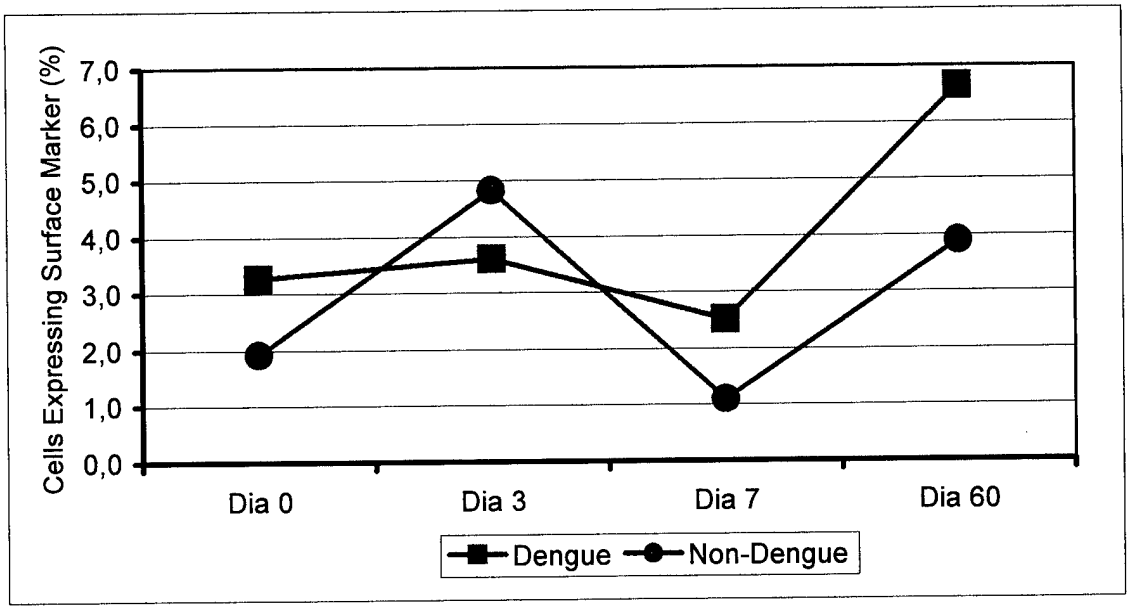


Figure 15 – Production of IL-1β by monocytes on both Dengue^{+} and non-Dengue^{+} patients.



CONCLUSIONS

Seventy-six percent of patients with fever were positive for Dengue. The results presented here suggest a central role for $CD8^+$ T cells and confirm the importance of Th1 cytokines in primary infections with Dengue virus. Although, leukopenia (specially on $CD3^+CD4^+$ T cell population) was a hallmark among $Dengue^+$ patients, levels of $CD3^+CD8^+$ T cells were higher on these patients. Our results also demonstrates a broad activation of both $CD4^+$ and $CD8^+$ T cells and NK cells on days 0, 3 and 7.

The analysis of cells producing cytokines showed a predominance of cells secreting Th1 cytokines (IFN-γ) on days 0, 3 and 7. $CD4^+$ T cells producing IFN-γ were upregulated on days 0, 3 and 7, reaching their peak on day 3. It also revealed an upregulation of on the production of TNF-α and IL-1β by monocytes, interestingly these two cytokines are associated with febrile manifestations and cells producing then were present at a higher concentration on days 0 and 7 but low on day 3. Although, not fully understood on day 3, activated NK cells ($CD56^+CD69^+$) were also down regulated if compared to days o and 7.

The results presented here support the idea that cytotoxic T cell responses associated with a strong Th1 response are part of the protective response against primary Dengue infection.

ADDITIONAL REPORTS

Field Evaluation of the Lethal Ovitrap system for control of *Aedes* mosquitoes.

A field test of the ovitrap was performed from February to May 1998 at four sites in Baixada Fluminense within the Rio de Janeiro area of Brazil. At each site, 30 houses were selected for participation in the study. Sixty received the lethal ovitrap treatment and ad additional 60 houses were selected to serve as untreated controls. Each treated house had 10 lethal ovitraps with strips impregnated with insecticide (deltamethrin 0.9 mg per strip). Five traps were placed inside the house and 5 traps outside the house. Each week 10 houses were examined, and the adult mosquitoes were collected. Prior to placing the lethal ovitraps at each house (both the treated and control), samples were taken of all mosquitoes found in the houses. Mosquitoes were collected for a ten minute period, using flashlights and battery operated aspirators. All ovitraps were checked monthly, during which time, the strip and the hay water solution were replaced. In addition, larvae and pupae found in the ovitraps were collected and identified to determine the effect of lethal ovitraps on natural *Aedes aegypti* and *Aedes albopictus* populations. Results from this test indicate that the lethal ovitraps significantly reduced the number of dengue vectors within the treated homes compared to the untreated control homes.

Drug Resistance Patterns in Parasitic Disease.
Mutation analysis of CG2- chloroquine resistance related gene in *Plasmodium falciparum* isolates from the Brazilian Amazon region-evidence of clonal expansion of chloroquine resistant parasite.

The first reports 40 years ago describing chloroquine resistance in *P. falciparum* pointed to an independent appearance in two foci. Strains

appearing in Southeast Asia spreading to Africa on the one hand, and in South America, on the other. Circumstantial evidence led to the belief that the over expression or mutations of a P-glycoprotein (pfmdr1 gene) was the cause of chloroquine resistance. However, genetic cross analysis between resistant and sensitive strains did not show segregation of mutations in the pfmdr1 loci. A recently published paper demonstrates a strong correlation between a specific set of polymorphisms in the cg2 gene of *P. falciparum* strains from Southeast Asia and Africa and chloroquine resistance. The data on the only South American strain in this discussion shows a different set of polymorphisms. Analysis of 18 fresh cultured-stable *P. falciparum* isolates from the Brazilian Amazon region showed in vitro resistance to chloroquine and quinine. The cg2 repeats and mutation pattern were similar to the South American strain 7G8. This data could indicate that chloroquine resistance in the Brazilian Amazon is a clonal expansion of a single resistant parasite and could point to a different mechanism of resistance in South American strains.

A comparison of the in vivo and in vitro activity of Pentostam of Chinese origin and Glucantime against *Leishmania brasiliensis* isolated from patients with cutaneous Leishmaniasis in Vitória, ES, Brasil.

The question of why some cutaneous Leishmaniasis patients treated with a standard course of antimonials sometimes cure and others do not is still ongoing. Most concerned scientists agree that the main causes are a combination of the intrinsic resistance of the parasites to the drugs used and the immune state of the patient.

Especially in the case of *L. brasiliensis*, it is essential to achieve a complete cure in which all parasites are eliminated from the body, rather than just the disappearance of the symptoms. It is widely hypothesized that the appearance of mucocutaneous Leishmaniasis is due to the reactivation of

quiescent parasites up to 10 or 15 years after a cutaneous infection. Appropriate treatment is crucial.

The in vitro resistance of parasites isolated from patients treated with standard courses of either glucantime or pentostam was examined. These patients were divided into two groups, cured or non-cured, according to the outcome of the treatment.

It was clearly shown that parasites from cured patients show low IC50s. A correlation was also established between *Leishmania* isolated from non-cured patients (treated with either glucantime or pentostam) and higher in vitro resistance of the parasite to the drug used for treatment. All, but one of these parasites showed elevated IC50s when compared to those isolated from patients who cured. In the case of NDI021, the treatment failed for reasons other than the intrinsic parasite resistance to the drug. Although, according to responses patients gave in questionnaires, they took their prescribed medication, non-compliance cannot be ruled out. The patients' immunological responses were not assessed. Sensitivity of *Leishmania* to an antimonial drug as tested in vitro appears to be an essential feature in the failure to cure patients.

Higher resistance to one drug does not imply higher resistance to the other drug. NDI027 and NDI028 showed high glucantime IC50s, but only NDI028 has an equally high IC50 for pentostam. NDI029 and NDI041 both demonstrate a lack of sensitivity to pentostam in vitro but have IC50s for glucantime that are equivalent to parasites from patients cured with glucantime. This could indicate a different mechanism of resistance specific to the compound drug rather than directed against antimony, itself.

Evaluation of leishmania secreted antigens by ELISA as a Diagnostic Tool

Due to the fact that the reagents and plastic supplies only arrived in Vitória only in February 1999 there is no specific data to report. However during 1998 there was This project is still in progress because the supplies to develop the ELISA, including the antigens, arrive in the lab only in February 1999. Since we got almost all the supplies we started the ELISA. So far, we had analyzed 96 serum from cutaneous leishmaniasis patients and 24 healthy individuals. The results showed not satisfactory suggesting that something was not working well. The serum from healthy patients presented high OD comparing to leishmaniasis patients serum. We had checked all the supplies and we concluded that the antigen was not working. This antigen needs to be used up to 3 months and the one that we had received was old (9 months). We are now waiting for new batch.

The Leishmania lab develop diagnosis of cutaneous and visceral leishmaniasis of volunteers patients that coming to the lab. In 1998, we had evaluated 47 patients with cutaneous lesions and 82 patients presenting symptoms related with visceral leishmaniasis such as fever and hepatoesplenomegaly.

The diagnose of cutaneous leishmaniasis was developed by biopsy of cutaneous lesions. One piece was used to do the imprint and the other one to develop the culture. The stained imprints were analyzed very carefully to detect the parasite.

Patients with visceral leishmaniasis symptoms were submitted to bone marrow aspirate. The diagnose was done microscopically on stained smears from bone marrow aspirates. Samples from skin biopsy and bone marrow aspirate were also cultured and examined afterwards. The culture of the leishmania promastigotes was developed using Schneider's medium and NNN with 20% of serum fetal calf. The culture was checked every day until the parasites grow. The parasites were cultured until a high number of them are reached. The parasites are harvested, washed many times with PBS and maintained in liquid nitrogen.

We had cultured and isolated 30 leishmania strains from patients with cutaneous and visceral leishmaniasis. The liquid nitrogen cryobank of strain is kept on.

We had also collected a blood sample from each patient before and after the treatment. The serum were separated and kept on freezer –70 C which had been used to develop the ELISA project.