

# The Cellular Immune Status of HBsAg-Positive Carriers in Malaysia

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Viral infections can cause dramatic changes in the number and proportion of peripheral blood lymphocyte subsets. Two infections with significant effects on blood lymphocytes are infections by the human immunodeficiency virus (HIV) and by the Epstein-Barr virus (EBV). Infection with HIV causes a profound decrease in CD4<sup>+</sup> T-cells<sup>1</sup> while infection with EBV leads to an expansion of CD8<sup>+</sup> T-cells.<sup>2</sup>

The cellular immune status of hepatitis B patients is known to differ from that of healthy individuals.<sup>3</sup> Changes in blood lymphocyte subsets occur with different phases of infection.<sup>4,5</sup> Data for peripheral blood lymphocytes during HBV infection are limited and contradictory.<sup>6-9</sup>

This study examines lymphocyte subsets in the peripheral blood of HBsAg carriers and their relationship to the HBV infectivity markers HBV-DNA, HBeAg/anti-HBe status and serum alanine transaminase (ALT). The lymphocyte subsets studied were T cells (CD3), B cells (CD19), CD4 cells, CD8 cells, Natural Killer (NK) cells (CD3<sup>-</sup>CD16<sup>+</sup>/CD56<sup>+</sup>) and the CD4/CD8 ratio.

**SUMMARY** The percentage of lymphocyte subsets from the peripheral blood of healthy adults and hepatitis B surface antigen (HBsAg) carriers were analyzed by flow cytometry. The five lymphocyte subsets studied were:- T (CD3) cells, B (CD19) cells, CD4 cells, CD8 cells, Natural Killer (CD3<sup>-</sup>CD16<sup>+</sup>/CD56<sup>+</sup>) cells (NK cells) and the CD4/CD8 ratio. The percentage (mean  $\pm$  SD) for the five lymphocyte subsets from the healthy adults were (67.5  $\pm$  8.5)%, (12.4  $\pm$  4.5)%, (35.5  $\pm$  7.8)%, (36.8  $\pm$  8.5)%, (17.9  $\pm$  8.1)% and 1.1  $\pm$  0.6, respectively. HBsAg carriers positive for HBV-DNA had a lower CD4/CD8 ratio than the healthy population (P=0.030). The percentage of CD8 cells in HBsAg carriers increased significantly (r=0.28; P=0.019) with an increase in ALT levels but the values remained within normal range. The percentage of NK cells and CD4/CD8 ratio in HBsAg carriers positive for anti-HBe were higher than HBsAg carriers negative for anti-HBe (92% of which are HBeAg positive) (P=0.045 and P=0.035, respectively). The CD4/CD8 ratio in HBsAg carriers negative for anti-HBe (92% positive for HBeAg) was also lower than in the healthy population (P=0.042). HBsAg carriers positive for HBV-DNA, HBeAg and raised ALT levels had a lower CD4/CD8 ratio than did the healthy population. The lower ratio was due to an increase in the percentage of CD8 cells. This suggests an activated immune response triggered by the infection in an attempt to clear the virus. HBsAg carriers with normal ALT levels and who are negative for HBV-DNA may be in a state of tolerance.

## MATERIALS AND METHODS

### Study Population

Blood was collected from 212 healthy volunteers ranging in age from 18-71 years. Healthy volunteers negative for HBsAg included blood donors, medical faculty staff and students. This group was made up of 74 Malays (37 males, 37 females), 77 Chinese (38 males, 39 females) and 61 Indians (37 males, 24 females). Blood from 120 HBsAg

positive carriers ranging in age from 17-70 years and from the healthy volunteers was collected from 1993-1994. Individuals were considered

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HBsAg carriers if they were found to be HBsAg positive for more than six months on repeat testing. Carriers comprised 43 Malays (35 males, 8 females), 67 Chinese (40 males, 27 females) and 10 Indians (6 males, 4 females).

Healthy volunteers and HBsAg carriers testing positive for the human immunodeficiency virus (HIV) were excluded, as were pregnant females. The healthy donors had not taken medication during the 2 weeks preceding the sampling. Samples were collected between approximately 8:00 am and 12:00 noon local time.<sup>10</sup> Blood samples were drawn simultaneously for flow cytometric analysis, serology for HIV and hepatitis B and haematological analysis.

#### Detection of HIV & HBV Markers

The samples were analyzed for HIV using the ABBOTT recombinant HIV-1/HIV-2 enzyme immunoassay. HBV testing was done using the AUSRIA II-125 'sandwich' radioimmunoassay for HBsAg and ABBOTT HBe (rDNA) 'sandwich' radioimmunoassay for the HBeAg/anti-HBe. Plasma HBV-DNA was detected by dot-blot hybridization with chemiluminescent detection.<sup>11</sup> Plasma ALT levels were determined in a routine biochemistry laboratory; levels of > 40 U/l for males and > 31 U/l for females were considered raised.

#### Flow Cytometric Analysis

The immunophenotype of lymphocytes was determined by dual immunofluorescence flow cytometry. Table 1 shows the combinations of fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated antibodies used to determine the expression of each lymphocyte antigen or antigen combination.

Venous blood was collected in tubes containing EDTA. Four

microlitres of each monoclonal reagent pair were added to 100 µl of whole blood. The mixture was gently mixed and incubated at room temperature (27°C) for 20 minutes in the dark. FACS brand lysing solution was added (2 ml of 1X) and the mixture incubated for a further 10 minutes at room temperature in the dark. The white cells were pelleted at 300 × g for 5 minutes at room temperature. The pellet was washed two times in phosphate buffered saline (8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCL; pH 7.4) before being resuspended in 1 ml phosphate buffered saline containing 1% paraformaldehyde.

The lymphocyte subsets were analyzed on a FACScan (Becton Dickinson Immunocytometry Sys-

tems, San Jose, CA) flow cytometer. The total white cell count was measured by a Coulter Counter (model JR) and the percentage of lymphocytes was obtained by a manual differential count of a hundred white cells on Wright stained blood films. SimulSET analysis software was programmed to measure a minimum of 10,000 white cells at the onset of the measurement. An analysis gate that included at least 95% of all lymphocytes was established by LeucoGATE reagent (CD45/CD14). A minimum of 2,000 lymphocytes were acquired from each tube with less than 10% contamination by debris, granulocytes or monocytes.

The delineating markers for determining cells positive and negative for any reagent were set by

Table 1. Panel of reagents.

Antigen	Antibody/Fluorochrome (Simultest reagent)
CD45 (Leucocytes) CD14 (Monocytes)	Anti-CD45/FITC Anti-CD14/PE (Simultest LeucoGATE)
Murine keyhole limpet Haemocyanin (KLH)	Murine monoclonal antibodies IgG <sub>1</sub> , IgG <sub>2</sub> antibody (Simultest Control)
CD3 (All T cells) CD19 (All B cells)	Anti-CD3/FITC Anti-CD19/PE (Simultest T & B cells Test)
CD4 (T helper/inducer cells) CD8 (T suppressor/cytotoxic cells)	Anti-CD4/FITC Anti-CD8/PE (Simultest CD4 & CD8 cells Test)
CD3 <sup>-</sup> , CD16 <sup>+</sup> and/or CD56 <sup>+</sup> (Natural killer cells)	Anti-CD3/FITC Anti-CD16 + CD56/PE (Simultest CD3 & CD16 + 56 cells Test)
FITC : Fluorescein isothiocyanate PE : Phycoerythrin	

SimulSET software using directly conjugated antibodies of irrelevant specificity (Simultest Control) as negative controls. Each two colour immunophenotype analysis was run and analyzed with the help of SimulSET software that determined the lymphocyte gate and marker settings. The results were expressed as a percentage of total lymphocytes.

### Quality Control

The FACScan was calibrated with fluorescent beads (CalIBRITE) and AutoCOMP software monthly. Besides the use of a single protocol and instrument throughout the study, an internal consistency control<sup>12</sup> way used, requiring %T + %B + %NK approximated 100% ± 5%.

### Statistical Analysis

Differences between races were analyzed nonparametrically by the Kruskal-Wallis test. The Mann-Whitney U-test was used to estimate the homogeneity between race and the null hypothesis of no difference was rejected at 5% probability. The influence of sex was evaluated by the Mann-Whitney U-test for each lymphocyte subset. Where there was a racial difference in subset distribution, the influence of sex was tested for each individual race using the Mann-Whitney U-test. Each lymphocyte subset was also analyzed for trends by age and ALT levels using simple linear regression. The various parameters of HBsAg carriers were compared to those of the healthy population using the Mann-Whitney U-test.

### RESULTS

Some differences between races, sex and age in the levels of the lymphocyte subsets for healthy Malaysian adults were shown statistically but were minor and of doubtful clinical significance.<sup>13</sup> The combined reference ranges were

therefore used. The parameters studied in the 120 HBsAg carriers are summarized in Fig. 1. The percentage of lymphocyte subsets for each parameter is shown in Table 2.

Overall, there was no significant difference between the healthy population and HBsAg carriers. However, when the HBsAg carriers were subdivided by parameter, significant differences emerged. The CD4/CD8 ratio in HBsAg carriers positive for HBV-DNA was significantly lower than that in healthy volunteers ( $P=0.030$ ). HBsAg carriers negative for HBV-DNA did not differ significantly from healthy controls in CD4/CD8 ratios ( $P=0.15$ ). Ninety-two percent of HBsAg carriers negative for anti-

HBe were positive for HBeAg. HBsAg carriers positive for anti-HBe had a significantly higher CD4/CD8 ratio than did HBsAg carriers negative for anti-HBe ( $P=0.035$ ). The CD4/CD8 ratio of HBsAg carriers positive for anti-HBe was not significantly different from that in healthy volunteers ( $P=0.53$ ). However, HBsAg carriers negative for anti-HBe had a lower CD4/CD8 ratio than did the healthy population ( $P=0.042$ ). The percentage of NK cells in HBsAg carriers positive for anti-HBe was significantly higher than those negative for anti-HBe ( $P=0.045$ ). The percentage of CD8 cells in HBsAg carriers as a whole showed a significant rise ( $r=0.28$ ;  $P=0.019$ ) with increased ALT levels

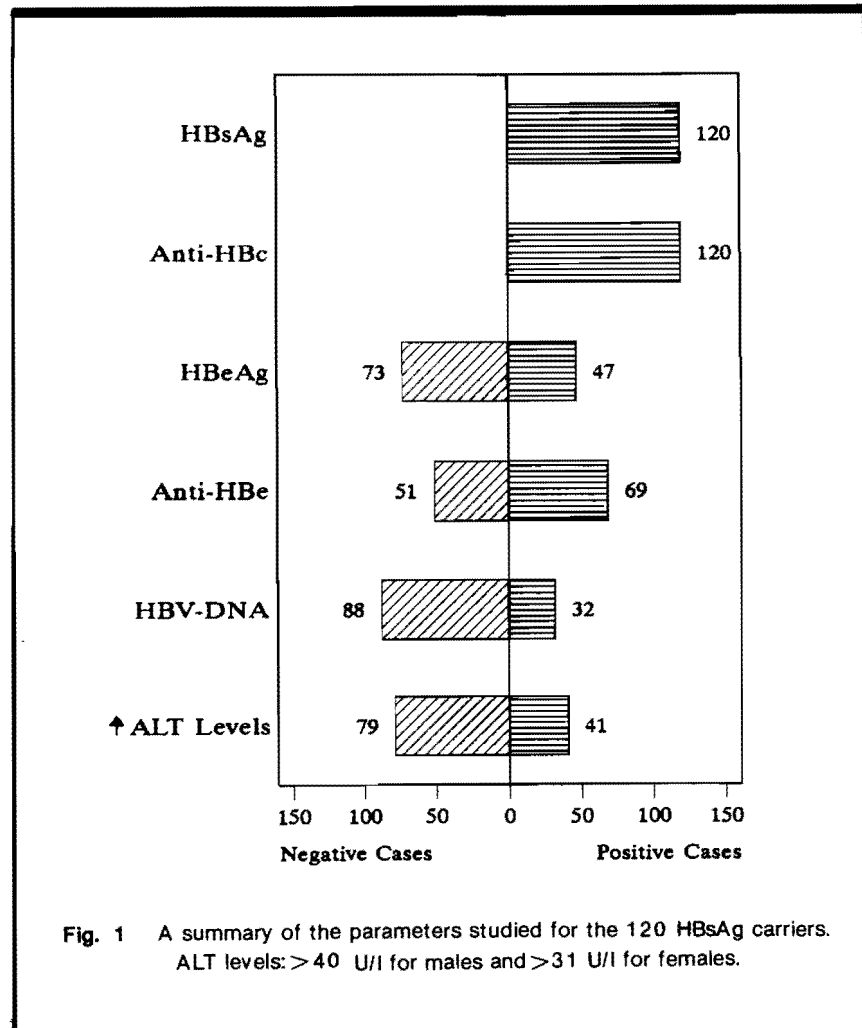


Fig. 1 A summary of the parameters studied for the 120 HBsAg carriers. ALT levels: >40 U/l for males and >31 U/l for females.

**Table 2.** Peripheral blood lymphocyte subsets of healthy volunteers and HBsAg carriers.

	Lymphocyte subset					
	T cells (%)	B cells (%)	CD4 cells (%)	CD8 cells (%)	CD4/CD8 (%)	NK cells (%)
Healthy	67.5 ± 8.5	12.4 ± 4.5	35.5 ± 7.8	36.8 ± 8.5	1.1 ± 0.6	17.9 ± 8.1
HBsAg <sup>1</sup> (+)	66.6 ± 9.5	12.6 ± 4.9	34.8 ± 9.2	37.5 ± 10.0	1.0 ± 0.5	18.3 ± 9.6
a. HBV-DNA (+)	66.3 ± 11.9	13.2 ± 5.0	32.8 ± 9.5	39.5 ± 10.5	0.9 ± 0.4*	19.7 ± 12.6
b. HBV-DNA (-)	66.7 ± 8.6	12.4 ± 4.9	35.6 ± 9.0	36.8 ± 9.8	1.1 ± 0.5	17.8 ± 8.2
c. HBeAg (+)	67.1 ± 10.6	13.6 ± 4.9	33.3 ± 9.9	37.9 ± 9.1	0.9 ± 0.4	17.3 ± 11.3
d. HBeAg (-)	66.2 ± 8.8	11.9 ± 4.9	35.8 ± 8.6	37.3 ± 10.7	1.1 ± 0.5	19.0 ± 8.2
e. Anti-HBe (+)	66.3 ± 8.9	11.9 ± 4.9	35.6 ± 8.6	36.5 ± 10.6	1.1 ± 0.5	19.2 ± 8.3 <sup>#</sup>
f. Anti-HBe (-)	66.9 ± 10.5	13.5 ± 4.9	33.7 ± 9.8	38.9 ± 9.1	0.9 ± 0.4 <sup>§</sup>	17.1 ± 11.0
g. ALT	67.4 ± 9.1	12.7 ± 5.6	33.6 ± 9.4	39.1 ± 11.1	0.9 ± 0.4	16.7 ± 8.4
h. Normal ALT	66.1 ± 9.8	12.4 ± 4.5	35.7 ± 8.8	36.5 ± 9.2	1.1 ± 0.5	19.3 ± 9.9

Results are expressed as percentage of the total lymphocytes (except for CD4/CD8 ratio) in mean ± standard deviation (SD).

<sup>1</sup> : HBsAg carriers as a whole without classification into various parameters.

a - h : The various parameters studied for the HBsAg carriers

(+) : positive

(-) : negative

ALT: raised ALT (>40 U/l in males and >31 U/l in females)

\* P=0.030 when compared to healthy volunteers

<sup>#</sup>P=0.045 when compared to anti-HBe negative HBsAg carriers

<sup>§</sup>P=0.035 when compared to anti-HBe positive HBsAg carriers and P=0.042 when compared to the healthy volunteers.

by linear regression analysis (Fig. 2). However, this increase remained within normal range. No significant difference between the carriers and healthy volunteers were noted for the other subsets.

## DISCUSSION

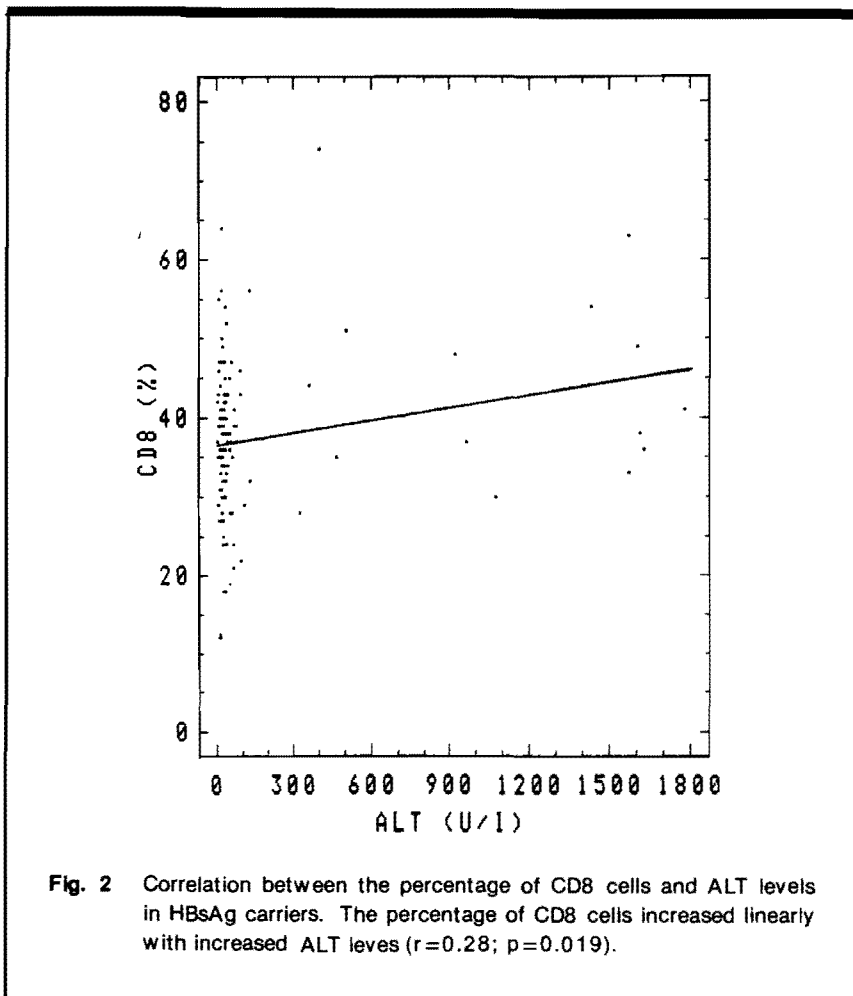
There was no difference in the percentage of T and B cells in HBsAg carriers compared to healthy controls despite the fact that HBV infection causes antibody and cell mediated immune responses.<sup>14,15</sup> The changes in T and B cells during HBV infection could occur in non-proliferating cells.

The NK cells are thought to modulate early HBV infection before T and B cells are primed.<sup>16</sup>

In this study, we did not find any significant changes in the percentage of NK cells in HBsAg carriers positive for HBeAg and HBV-DNA. However, the percentage of NK cells was higher in HBsAg carriers positive for anti-HBe than in carriers negative for anti-HBe (P=0.045; 92% of which are HBeAg positive). This could be due to an accumulation of NK cells in the liver during the early phase of infection. Alternatively, the NK cells could have been killed during early defence against infection. As seroconversion from HBeAg to anti-HBe occurs, the role played by NK cells is over by cytotoxic T cells. Hence, more NK cells would be released into the peripheral circulation or regenerated. The percentage of NK

cells did not change with increases in ALT levels. This is in concordance with the findings of Chemello *et al.*<sup>16</sup> who noted an insignificant relationship between NK cell cytotoxic activities and raised ALT levels and suggests that NK cells do not contribute to hepatolysis during HBV infection.

We also found no significant changes in the percentages of CD4 and CD8 cells in asymptomatic HBsAg carriers (normal ALT levels).<sup>9</sup> By measuring the transferrin receptor, IL-2, and HLA-DR on the surface of T cells, Raptopoulou-Gigi<sup>9</sup> observed an increase in activated T cells subsets. A proportion of CD4 and CD8 cells could be activated during HBV infection



which does not increase the number of cells.

There was no significant difference in the percentage of CD4 cells, CD8 cells and CD4/CD8 ratio in HBsAg carriers positive for HBeAg compared to the healthy controls. This is in contrast to a report from Taiwan<sup>17</sup> where a decrease in the CD4/CD8 ratio was observed in HBeAg positive carriers. The decrease was due to an increase in CD8 cells. However, our observation that a decrease in CD4/CD8 ratio in HBsAg carriers positive for HBV-DNA ( $P=0.030$ ) is in concordance with Thomas *et al.*<sup>6</sup> The percentage of CD8 cells was observed to increase linearly with ALT levels ( $P=0.019$ ) although the increase remained within normal range.

A possible explanation for our observations is that the decrease in the CD4/CD8 ratio in HBsAg carriers with active viral replication (as indicated by the presence of plasma HBV-DNA) could be due to an increase in cytotoxic CD8 cells. Cytotoxic CD8 cells have been shown to attack hepatocytes with HBcAg on their surface.<sup>15,18</sup> Lysis of hepatocytes would release both ALT and HBV-DNA. In acute self-limiting hepatitis B, an increase in ALT precedes the elimination of HBV.<sup>19</sup> This suggests that HBsAg carriers with reduced CD4/CD8 ratios may be in the process of recovery (seroconverting to anti-HBe). This hypothesis could be further tested by carefully following up this group of patients. HBsAg

carriers without an increase in ALT levels could be in a tolerance state with the HBV. Cytotoxic CD8 cells are not attacking hepatocytes. The compromised state is also indicated by a low level of HBV replication (absence or non-detectable HBV-DNA in the blood). Integration of HBV-DNA into the hepatocyte genome would occur if the condition persisted.<sup>20</sup>

HBsAg carriers can seroconvert for no apparent reason. A functional analysis of lymphocyte subsets would be of additional value to determine the interaction between low level antigenic challenge and immune cell responses which do not result in a deviation in the percentage of lymphocyte subsets.

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