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Lymphocyte Subpopulation Reference Ranges for Monitoring Human Immunodeficiency Virus-Infected Chinese Adults

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Two hundred eight healthy human immunodeficiency virus (HIV) type 1- and HIV type 2-seronegative Chinese adults (78 males and 130 females; mean age, 32 years; age range, 18 to 71 years) were analyzed for lymphocyte subsets by a standardized and quality-controlled flow cytometric immunophenotyping technique. While the leukocyte differential values were comparable to those found in studies of Caucasians, the means, medians, and 95% reference ranges of lymphocyte subsets were very different. The 95% reference ranges in absolute counts per microliter of whole blood (percentage of lymphocytes) for $CD3^+$, $CD3^+$ $CD4^+$, $CD3^+$ $CD8^+$, $CD3^-$ $CD19^+$ (B), and $CD3^-$ with $CD16^+$ and/or $CD56^+$ (NK) cells were 672 to 2,368 (54.8 to 83.0%), 292 to 1,366 (23.1 to 51.0%), 240 to 1,028 (17.9 to 47.5%), 82 to 560 (5.1 to 20.8%), and 130 to 938 (7.1 to 38.0%), respectively. $CD3^+$ $CD4^+$ cells showed significant sex difference (for males, mean of 702 [34.8%] and standard deviation of 258 [7.5%]; for females, mean of 728 [37.3%] and standard deviation of 254 [7.4%]) as well as an increase with age of 42 (1.6%) per decade. Investigations of the NK cell population did not show similar findings. Classification of HIV disease, treatment, and prophylactic regimens based on studies which relied heavily on estimations of lymphocyte subsets alone should be used with special caution for Chinese patients. Provided that adequate quality control measures are taken to ensure comparability of data, we recommend that these ranges be used on a day-to-day basis in laboratories that have not yet established their own reference ranges.

The World Health Organization has estimated that more than 90% of a world total of 30 to 40 million cases of human immunodeficiency virus (HIV) infection by the year 2000 will be in developing countries (9). In South and Southeast Asia, the prevalence of HIV infection is much higher than the number of AIDS cases would suggest. The World Health Organization estimates that more than 2.5 million adults in this area were infected with HIV, but only 14,527 AIDS cases were reported by the end of 1994 (24). Present knowledge concerning the staging of disease, monitoring of disease progression, and initiation of therapeutic regimens depends heavily on determination of peripheral lymphocyte subpopulations (4, 17). Normal ranges of lymphocyte subpopulations in adults have been established for Caucasians, but little data are presently available for immunophenotype assessment of HIV-infected Chinese adults. Early studies of a small number of blood donors of Chinese ethnic origin in the United States, using only T3, T4A, T8, and Leu 11 as markers, documented possible significant differences from the Caucasian population (19). In anticipation of the impending wave of HIV infections for the 1.2 billion Chinese residing in Asia, we recognized the urgent need to have a set of accurate reference range values ready for our indigenous population. This present study used a standardized and quality-controlled flow cytometric procedure to survey healthy Chinese adults and establish lymphocyte subpopulation reference ranges which are relevant to immunological monitoring of HIV disease.

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MATERIALS AND METHODS

Study population. Healthy adults of Chinese ethnic origin who worked in various clinical laboratories in the Institute of Pathology, Department of Health, Hong Kong, were invited to participate in the study. A questionnaire given each participant asked if they had a vaccination within the past 3 months, had an infection in the past 4 weeks (including viral, bacterial, fungal, and other pathogens), had taken antibiotics in the past 4 weeks, had been hospitalized within the past 2 years, or had taken medication, including analgesics, nonsteroidal anti-inflammatory agents, antiulcer drugs, antihypertensive drugs, and other cardiovascular drugs. Subjects who reported a positive history for any of these items were excluded from the study. All participants were seronegative for HIV type 1 (HIV-1) and HIV-2 by enzyme-linked immunosorbent assay. Personnel who supplied informed consent had samples collected between approximately 9:00 a.m. and 12:00 p.m. Blood samples were drawn simultaneously for the hematology laboratory and for flow cytometric analysis. Absolute counts of cells were obtained by multiplication of the percentage of lymphocytes by the leukocyte differential obtained from a simultaneous blood sample analyzed with an automated hematological instrument (Coulter MAXM; Coulter Corporation, Miami, Fla.).

Reagents, cell preparation, and staining. A standardized method using a recommended panel (1, 5, 16) of two-color combinations of fluorescein isothiocyanate- and phycoerythrin-conjugated monoclonal antibody reagents obtained from a single manufacturer (Becton Dickinson, San Jose, Calif.) was used to determine the expression of each antigen or antigen combination. All samples were processed within 6 h of blood collection (7). Briefly, 3 ml of whole blood was collected in EDTA. For each sample, 20 μl of each monoclonal reagent pair (Simultest) was added to 100 μl of well-mixed anticoagulated whole blood in test tubes (12 by 75 mm). The mixture was gently vortexed and incubated for 15 min at room temperature in the dark. After incubation, 2 ml of 1× fluorescence-activated cell sorter (FACS) lysing solution was added, and the mixture was immediately vortexed and then incubated for 10 min at room temperature in the dark. The tubes were centrifuged at 300 × *g* for 5 min at room temperature, and the supernatant was aspirated or decanted, leaving behind approximately 50 μl of residual fluid; disturbance to the pellet was avoided during the process. Two milliliters of 1× phosphate-buffered saline (PBS) with 0.1% azide (filtered before use) was added to resuspend the pellet. After gentle vortexing and centrifugation at 200 × *g* for 5 min at room temperature, the supernatant was aspirated or decanted as before, and the pellet was resuspended in 0.5 ml of 1.0% paraformaldehyde in either sheath fluid or PBS with 0.1% azide. Samples were stored at 2 to 8°C in the dark and analyzed within 24 h of lysing and staining.

Flow cytometry analysis and quality control. Data acquisition was performed on configured FACScan (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) flow cytometers. The daily instrument setup was performed with

TABLE 1. Age and sex distributions for the study population

Age (yr)	No. (%):		
	Female	Male	Total
10–19	0 (0)	1 (1.2)	1 (0.5)
20–29	53 (40.8)	22 (28.2)	75 (36.1)
30–39	45 (34.6)	22 (28.2)	67 (32.2)
40–49	23 (17.7)	17 (21.8)	40 (19.2)
50–59	5 (3.8)	14 (17.9)	19 (9.1)
60–69	2 (1.5)	2 (2.6)	4 (1.9)
70–79	2 (1.5)	0 (0.0)	2 (1.0)
Total	130 (62.5)	78 (37.5)	208 (100)

CaliBRITE beads and AutoCOMP software. The determination of positive and negative cells for any combination of reagents was set by markers in the SimulSET software with directly conjugated antibodies of irrelevant specificity (Simultest control) as negative controls. Cursors were set by using isotype control so that <2% of the cells were positive. Each two-color immunophenotype analysis was run with the SimulSET software, which determined lymphocyte gate and marker settings and calculated singly or doubly positive events for each reagent pair, as previously described (13). Dot plot results for each two-color pair were analyzed.

Acceptance criteria for the lymphocyte gate included the following: ≤3% monocytes within the gate, ≤6% granulocytes within the gate, and ≤10% debris within the gate. A minimum of 10,000 total events were acquired by using the SimulSET software and the LeucoGATE reagent (CD45/CD14). A lymphocyte gate which recovered at least 95% of the lymphocytes with a lymphocyte purity of at least 90% was established. When the recovery and purity of lymphocytes within the gate did not meet the recommended minimum levels, the gate was redrawn. If the minimum levels still could not be obtained, the specimen was reprocessed. If this also failed, another specimen was requested. List mode data for all specimens analyzed were stored and allowed reanalysis of raw data, including redrawing of gates.

The remaining tubes were checked for reliability by adding the percentages of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ cells, which equaled the total percentage of $CD3^+$ cells within ±5%. The sum of the percentages of T cells ($CD3^+$), B cells ($CD19^+$ $CD3^-$), and NK cells ($CD3^-$ with $CD16^+$ and/or $CD56^+$) equaled the purity of lymphocytes in the gate ±5%, with a maximum variability of ≤10%.

Statistical analysis. The mean, median, and standard deviation were calculated. The 95% reference range for the data was the interval bounded by the lowest datum point below which lie 2.5% of the datum points and the highest datum point above which lie 2.5% of the datum points. The influences of sex and age were evaluated by analysis with the SPSS computer package (SPCC/PC+ software, version 2.0; SPSS Inc.). Levene's test for equality of variances was used to determine possible sex differences. A two-tailed P value of <0.025 was taken as indicating significance. The trends of change in individual markers with increasing age were analyzed by linear regression with a P value of <0.025 as determining statistical significance.

RESULTS

Of a total of 217 persons who volunteered for the testing, 9 were excluded (because of uncertain histories [2 persons] or recent infections [3 persons] and/or medications [4 persons]). Table 1 shows the distributions of sex and age for the 208 subjects considered evaluable for the study; there were 130 females and 78 males, with a mean age of 32 years (range, 18 to 71 years). The 20- to 40-year age group accounted for 68.3% of the study subjects. The leukocyte three-part differential count values are shown in Table 2, and their distribution curves are shown in Fig. 1. Comparison of the mean, median, standard deviation, and 95% reference ranges with those previously established by other means showed very similar results. This gave us an added measure to ensure that our subjects were normal as judged by the leukocyte differential counts. The distribution curves for both lymphocytes and granulocytes were basically Gaussian, while that for monocytes showed a slight skew towards higher values.

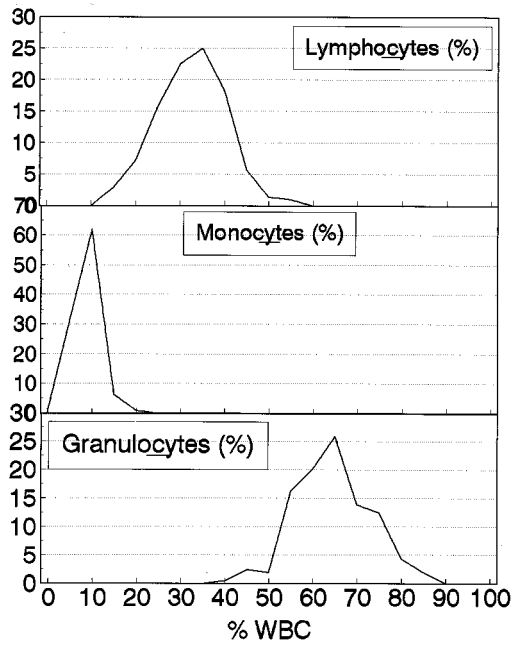

FIG. 1. Distribution of leukocyte (WBC) differential counts in the study population (n = 208).

The lymphocyte subset values are shown in Table 3, and the distribution curves for $CD3^+$ cells (total T cells), $CD3^+$ $CD4^+$ cells (T helper cells), $CD3^+$ $CD8^+$ cells (T suppressor cells), $CD3^-$ $CD19^+$ cells (B cells), and $CD3^-$ with $CD16^+$ and/or $CD56^+$ cells (NK cells) are shown in Fig. 2. Establishment of reference ranges for the $CD4^+$ and $CD8^+$ markers was also included to enable comparison with older methods, including immunoperoxidase staining techniques, which we previously used to monitor our HIV-infected patients and which measured only these single monoclonal markers and the $CD4^+$/$CD8^+$ ratio. Measurement of the $CD4^+$ marker alone overestimated the T helper-inducer lymphocyte subpopulation by 7.1% compared with the use of two markers ($CD3^+$ and $CD4^+$).

Analysis of possible gender influence for all markers showed no significant differences except for $CD3^+$ $CD4^+$, with females having a higher absolute number and percentage of lymphocytes than males (two-tailed $P < 0.025$). The absolute $CD3^+$ $CD4^+$ counts (percentages of lymphocytes) for males and females, respectively, were as follows: mean, 702 (34.8) and 738

TABLE 2. Leukocyte differential counts for the study population

Cell type	No./μl (%)			
	Mean	Median	SD	95% reference range
Lymphocytes	1,981 (30.6)	1,930 (31.0)	526 (7.7)	1,052–3182 (15.2–45.8)
Monocytes	466 (6.9)	420 (7.0)	275 (2.4)	230–868 (3.0–14.0)
Granulocytes	4,227 (62.4)	3,960 (62.0)	1,452 (8.3)	2,200–7816 (45.0–79.8)

TABLE 3. Mean, median, standard deviation, and 95% reference ranges of lymphocyte subsets in healthy Chinese adults

Antigen or cell type	% (no./μl)			
	Mean	Median	SD	95% reference range
$CD3^+$	69.0 (1,370)	69.2 (1,350)	7.7 (400)	54.8–83.0 (672–2,368)
$CD3^+$ $CD4^+$	36.4 (725)	36.0 (670)	7.5 (256)	23.1–51.0 (292–1,366)
$CD3^+$ $CD8^+$	29.7 (589)	29.1 (570)	7.2 (205)	17.9–47.5 (240–1,028)
$CD19^+$ $CD3^-$	11.1 (221)	10.8 (190)	3.9 (108)	5.1–20.8 (82–560)
NK cell	19.8 (394)	19.1 (340)	8.1 (194)	7.1–38.0 (130–938)
$CD38^+$	83.0 (1,646)	85.0 (1,655)	10.1 (444)	60.1–96.0 (874–2,629)
$CD8^+$ $CD38^+$	29.2 (576)	29.0 (575)	7.2 (187)	15.4–45.0 (220–1,041)
HLA-DR^+	42.3 (845)	43.0 (810)	10.2 (316)	25.0–60.7 (364–1,642)
$CD8^+$ HLA-DR^+	16.1 (323)	15.0 (290)	6.7 (164)	4.4–30.0 (60–690)
$CD4^+$	39.0 (785)	38.0 (730)	7.6 (262)	25.4–54.6 (330–1,508)
$CD8^+$	40.6 (805)	40.0 (780)	7.6 (247)	26.2–56.8 (420–1,358)
$CD4^+/CD8^+$ ratio	1.0	1.0	0.4	0.5–1.8
$CD3^+$ $CD4^+/CD3^+$ $CD8^+$ ratio	1.3	1.2	0.5	0.6–2.5

(37.3); standard deviation, 258 (7.5) and 254 (7.4); and standard error of the mean, 29.5 (0.9) and 22.3 (0.6). The distribution curves for these lymphocyte subsets were also Gaussian, except for that for B cells, which was slightly skewed to higher values. This skew was more pronounced for NK cells.

Of particular note was the presence of 32 (15.4%) and 33 (15.9%) subjects with $CD3^+$ $CD4^+$ counts of <500/μl and <29% lymphocytes, respectively, while 6 subjects (2.9%) had counts of <300/μl. If the single $CD4^+$ marker was used, the corresponding values were 10.7, 4.8, and 1.2%, respectively. No subject was found to have counts of <200/μl (<14%).

Figure 3 shows the distribution curves for the lymphocyte activation markers $CD38^+$, $CD8^+$ $CD38^+$, HLA-DR^+, and $CD8^+$ HLA-DR^+, which are useful for monitoring HIV disease. While normal curves were obtained for these markers, an apparent bimodal distribution with two separate peaks of

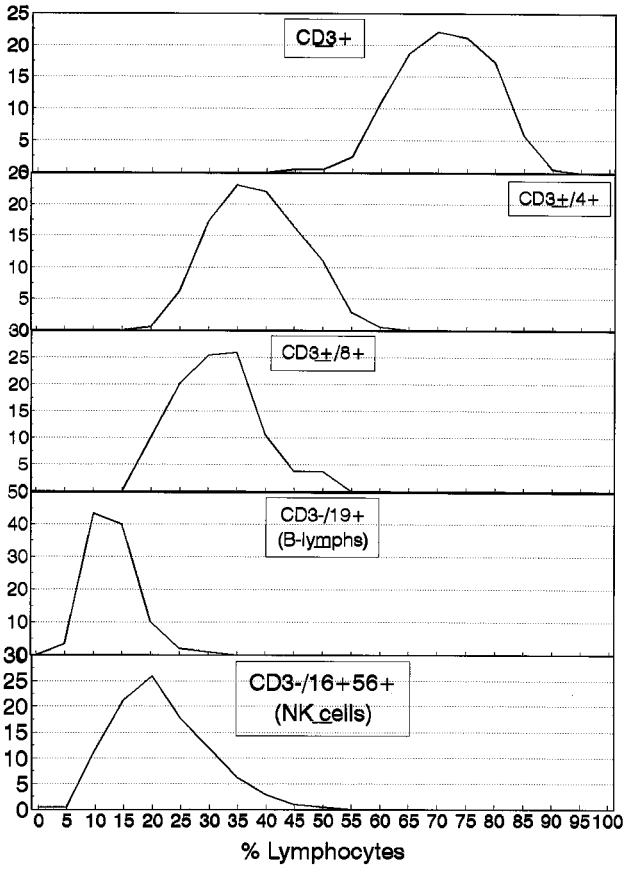

FIG. 2. Distribution of lymphocyte subsets ($CD3^+$, $CD3^+$ $CD4^+$, $CD3^+$ $CD8^+$, $CD3^-$ $CD19^+$ [B cells], and $CD3^-$ with $CD16^+$ and/or $CD56^+$ [NK cells]) in the study population (n = 208). The y axis shows the percentage of samples.

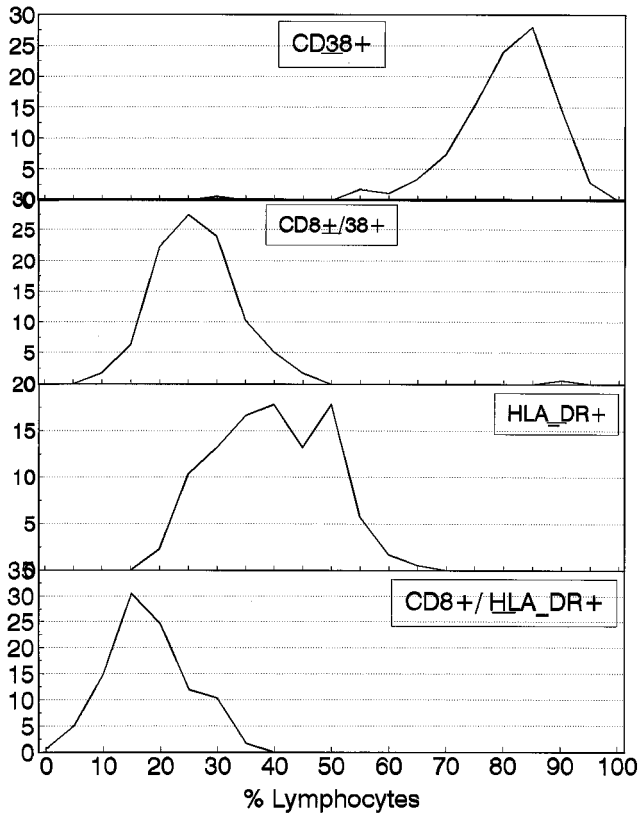

FIG. 3. Distribution of lymphocyte subsets ($CD38^+$, $CD8^+$ $CD38^+$, HLA-DR^+, and $CD8^+$ HLA-DR^+) in the study population (n = 208). The y axis shows the percentage of samples.

TABLE 4. Analysis of the effect of age on lymphocyte subpopulations[a]

Antigen or cell type	*a*	*b*	*P*
$CD3^+$	69.50	−0.0133	0.7850
$CD3^+$ $CD4^+$	30.89	0.1562	0.0008[b]
$CD3^+$ $CD8^+$	32.05	−0.0664	0.1420
$CD19^+$ $CD3^-$	9.62	0.0415	0.0918
NK cell	20.93	−0.0306	0.5490
$CD38^+$	92.19	−0.2691	0.0005[b]
$CD8^+$ $CD38^+$	33.56	−0.1290	0.0199[b]
HLA-DR^+	35.59	0.1983	0.0127[b]
$CD8^+$ HLA-DR^+	13.04	0.0897	0.0888
$CD4^+$	33.66	0.1569	0.0091[b]
$CD8^+$	43.57	−0.0859	0.1567
$CD4^+/CD8^+$	0.76	0.0077	0.0072[b]
$CD3^+$ $CD4^+/CD3^+$ $CD8^+$	0.93	0.0113	0.0004[b]

[a] Let $y = a + bx + e$ (e = residual). The null hypothesis is that age has no effect on the antigen or cell type (y) (i.e., $b = 0$).
[b] $P < 0.025$.

HLA-DR^+ cells at the 40 and 55% lymphocyte values was noted.

The results of analysis of a possible effect of age on lymphocyte subpopulation changes are shown in Table 4. Although at the $P < 0.025$ significance level, $CD3^+$ $CD4^+$, HLA-DR^+, and $CD4^+$ cells as percentages of lymphocytes appeared to increase, while $CD38^+$ and $CD8^+$ $CD38^+$ cells decreased, with increasing age, only the $CD3^+$ $CD4^+$ and $CD38^+$ correlations were significant at the $P < 0.005$ level. $CD3^+$ $CD4^+$ cells appeared to increase at a rate of 1.6% per decade in our study population, while $CD38^+$ cells decreased at a rate of 2.7% per decade. Correspondingly, the $CD3^+$ $CD4^+/CD3^+$ $CD8^+$ ratio showed an increase of 0.11 per decade. In particular, no significant increase in the NK cell population with increasing age was observed.

DISCUSSION

During the initial planning stages of our study, we were very concerned about the reproducibility and comparability of our test results, given the contradictory results of previous studies (14, 15). We acknowledged the problem of interlaboratory variations and have chosen a standardized protocol with good quality control procedures. We also endeavored to minimize and assess our laboratory variations by participation in College of American Pathologists surveys. Moreover, a local smaller-scale study, using similar reagents but different instrumentation (Coulter Profile II), which showed very comparable results gave added support to the results of our study. The effects of interlaboratory variations in instrumentation, monoclonal antibodies, and fluorochromes on flow cytometric immunophenotyping results have been studied. Gelman et al. (8), using data from 2 years of the flow cytometry quality assessment program of the Division of AIDS, National Institute of Allergy and Infectious Diseases, in the United States, reported that for HIV-positive specimens FACScans produced significantly lower percent $CD4^+$ values than EPICS-Cs or EPICS profiles and that for HIV-negative specimens fluorescein isothiocyanate was associated with significantly lower percent $CD4^+$ values than phycoerythrin or RD1. They remarked that differences were never larger than 2% and that regressions accounted for only 3 to 12% of the variability in their study. We have repeated our investigations with the same methodology and instrumentation for another, smaller group of healthy adults and have found similar results. Therefore, we agree that machine differences may not be significant as long as standardized and quality-controlled procedures are followed in the ergodic process of measurement of the antigens (20). We have also shown that in our study population, detection of the single marker $CD4^+$ alone overestimates by 7.1% (39.0 versus 36.4%) the T helper-inducer subpopulation compared with the use of a two-color (fluorescein isothiocyanate-phycoerythrin) assessment of cells ($CD3^+$ $CD4^+$).

Although there is the possibility of missing subjects who might be at their window period for seroconversion, our ongoing antibody screening program for HIV-1 and HIV-2 among blood donors as well as unlinked anonymous testing for risk groups (prison inmates and drug users) showed that HIV disease is probably present at very low levels in our general population (<0.001%) and in risk groups in Hong Kong (<1%). Therefore, it appears extremely unlikely that HIV disease could account for our low $CD4^+$ results. Other possibilities are subclinical hepatitis and/or mycobacterial infections; both infections are highly prevalent in the Chinese population and are known to depress T helper-inducer cells without affecting the helper/suppressor ratio (3, 12), and they were not completely excluded by our detailed history. This possibility, however, would have to be substantiated by further studies of our local population.

Similar immunophenotyping studies with Caucasians, using similar instrumentation, reagents, and procedures, showed various possible significant differences (20). While the differential leukocyte values were very similar, a comparison of the lymphocyte subset populations showed some noteworthy differences. The total T-cell ($CD3^+$) and B-cell ($CD3^-$ $CD19^+$) absolute counts as well as percentages of lymphocytes were low among our study population, while NK cell levels were high. Although the significant sex difference for $CD3^+$ $CD4^+$ cells as well as the trend of increasing percentage of $CD3^+$ $CD4^+$ cells with age seen in Caucasians was also observed among our Chinese subjects, we did not see any significant rise in NK cells ($CD3^-$ with $CD16^+$ and/or $CD56^+$) with age in our population. Two possible explanations can be given for this. First, our population could have been exposed to environmental agents, including various infectious agents, during the early stages of life and could have maintained a high NK cell population throughout adulthood. Second, a genetic difference could account for this difference. A study on the evolution of the lymphocyte subsets in our pediatric population would help to elucidate this point.

Prince et al. (19) used anti-Leu 11 as an NK cell marker to study 31 blood donors of Chinese ethnic origin in the United States and observed two distinct clusters. It was not stated whether these were recent migrants from China or persons of Chinese ancestry born in the United States. We used $CD3^-$ with $CD16^+$ and/or $CD56^+$ as an NK cell marker and studied an indigenous Chinese population, and we did not confirm the observations of Prince et al. (19). It is possible that migrant Chinese and ethnic Chinese born in the United States may have been exposed to different environmental agents during early childhood or that the heterogeneity of the different Chinese ethnic groups was not reflected in our present sample, which consisted mainly of southern Chinese, or the difference could be due to different NK cell markers being used.

The activation antigens, $CD38^+$ and HLA-DR^+ on $CD8^+$ lymphocytes, have been documented to be useful in monitoring HIV-1 disease in Caucasians (11). Our preliminary observations for HIV-1-infected Chinese showed that a subgroup of our patients may also display these markers during the course of HIV disease. An even better way to examine CD38 and HLA-DR expression is by using three-color immunofluores-

cence with CD3 to eliminate NK cells ($CD3^-$ $CD8^+$) that may contribute to the percentage of positive cells. In the future, the CD38 measure very well may be in terms of antibody binding sites and not percent fluorescence.

The present Centers for Disease Control and Prevention staging criteria for progression of HIV disease rely heavily on an accurate assessment of peripheral $CD3^+$ $CD4^+$ cells, expressed either as an absolute count or as a percentage of lymphocytes (6). Importantly, the initiation of antiretroviral therapy for persons with $CD4^+$ T-lymphocyte counts of $>500/\mu l$ (17) and prophylaxis against *Pneumocystis carinii* pneumonia for those with counts of $<200/\mu l$ have been recommended (4). With our present values, the former regimen would be started in at least 15.4% of our HIV-positive asymptomatic patients (stage A2). Notwithstanding the fact that zidovudine may be initiated early rather than late in the course of HIV disease (17), we think that there is a high chance that strict adoption of the criterion of <500 cells per μl for treatment would lead to overtreatment of our HIV-infected Chinese subjects with zidovudine. Moreover, 2.9% of our adult population would be classified as having idiopathic $CD4^+$ T lymphopenia if the criterion of <300 $CD4^+$ cells per μl was used (22). At present, we are uncertain whether repeated testing will result in a smaller percentage of such cases. Therefore, pending the availability of staging data which give a better correlation with clinical findings, we agree with the expanded World Health Organization case definition for AIDS surveillance (23) and would be hesitant to adopt strict Centers for Disease Control and Prevention definitions in our setting. Much therefore remains to be done to delineate the use of different surrogate markers to monitor progression of and guide therapy for HIV disease in the Chinese population.

In conclusion, we anticipate that, with our establishment of lymphocyte subset reference ranges, immunophenotype monitoring in future studies of HIV disease among Chinese patients will be greatly facilitated by data generated by comparable, reproducible, and quality-controlled flow cytometry technology (18, 21). Provided that such quality control measures are taken to ensure comparability of data, we recommend that these ranges be used on a daily basis in clinical and diagnostic immunology laboratories that have not yet established their own reference ranges. In particular, it will be important to study the maturational and developmental changes of lymphocyte subpopulations in Chinese subjects from infancy to adulthood and to compare these data with those for Caucasians (2, 10).

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