amide gel electrophoresis (PAGE) was performed with a 4 20% gradient polyacrylamide gel (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). The separated proteins were transferred to nitrocellulose membranes at 144 mA for 30 min, after which the membranes were probed with mouse anti-SIV p27 (kindly supplied by Dr. Sata of NIID) followed by anti-mouse IgG-alkaline phosphatase conjugate (New England BioLabs, Inc., Beverly, MA). Proteins were visualized by reactivity with NBT/BCIP (Roche Diagnostics Co., Indianapolis, IN). The concentration of SIV Gag p27 antigen in the cell extract was determined by a commercial antigen ELISA (SIV Core Antigen Assay kit, Coulter Corporation, Miami, Florida) as per the manufacturer's instructions.

Immunization of guinea pigs with rBCG-SIVGag

Guinea pigs were inoculated with either rBCG-SIVGag or BCG by the i.d. or oral route. For i.d. immunization, six guinea pigs were given a single inoculation of 0.1 mg of rBCG-SIVGag. Three separate control groups consisting of three animals each received i.d. inoculation with either (1) rBCG-pSO246 as a plasmid vector control, (2) BCG-Tokyo as a BCG vector control or (3) saline alone. Prior to oral immunization, fifteen guinea pigs were deprived of food and water overnight. The following day, 1 ml of 3% sodium bicarbonate was administered orally to each animal via a micropipette to neutralize stomach fluid, after which 500 μl of saline containing either 80 mg of rBCG-SIVGag (n = 6), rBCG-pSO246 (n = 3), BCG-Tokyo (n = 3) or saline alone (n = 3) was administrated by the same route. To flush the remaining antigens in their mouths and esophagi, saline (500 μl) was given orally to the animals after antigen ingestion. These procedures were performed on the animals under non-anesthetic conditions. Oral immunization was performed once a week for 2 consecutive weeks, providing a total dose of 160 mg of either rBCG-SIVGag, rBCG-pSO246 or BCG-Tokyo per animal.

Induction of a delayed type hypersensitivity (DTH) skin reaction

To investigate antigen-specific T cell immunity, DTH skin tests were performed at 8 and 50 weeks after immunization with either rBCG-SIVGag, rBCG-pSO246 or BCG-Tokyo. To evaluate SIV Gag-specific DTH responses, 10 μg of SIV Gag p27 protein (Advanced Biotechnologies Inc., Columbia, MD) per 100 μl of saline was injected i.d. into both immunized and non-immunized guinea pigs. To evaluate tuberculosis-specific DTH responses, 0.5 μg of PPD was administered by the same procedure. Saline (100 μl) was used as a negative control. After 24, 48 and 72 h, the diameter of each area of induration was measured.

Isolation of peripheral blood mononuclear cells (PBMC), spleen cells, intestinal intraepithelial lymphocytes (i-IEL) and lamina propria lymphocytes (LPL)

PBMC were separated from heparinized blood using Lympho-separ according to the manufacturer's instructions (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan). To isolate spleen cells, guinea pigs were sacrificed while under anesthesia with ketamine hydrochloride (Sankyo Co., Ltd., Tokyo, Japan), and their spleens were harvested. Spleen cells were prepared by gentle dispersion through a 70-μm nylon mesh (Becton Dickinson, Franklin Lakes, NJ). The preparations were treated with ACK lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA) for 1 min at room temperature to remove red blood cells, and the remaining cells were washed three times in PBS. Preparation of i-IEL was performed as previously described [27 29]. Briefly, large or small intestines were opened longitudinally and washed with PBS containing 1 mM DTT. The tissues were then placed in 20 ml of RPMI 1640 containing 1 mM EDTA in a 50-ml centrifuge tube and incubated for 20 min at 37 C with shaking. After incubation, the tube was shaken vigorously for 15 s, and the cell-containing medium was removed and saved. This process was repeated three times. To isolate LPL, the remaining intestinal tissues were treated with 0.5 mg/ml collagenase (Sigma Chemical Co., St. Louis, MO) and 1.0 mg/ml hyaluronidase (Sigma) for 30 min at 37 C with shaking, and the cell-containing medium was removed and saved. This process was repeated twice, and the harvested cells were then purified through a discontinuous 40/75% percoll gradient (Pharmacia, Uppsala, Sweden).

Antigen-specific T cell proliferative responses

PBMC were re-suspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 10 μg/ml gentamicin. The cells were cultured in triplicate wells at a density of 2 × 10^6 cells/ml in the presence or absence of antigen (20 μg/ml of either PPD or SIV Gag p27) in 96-well U-bottomed plates (Costar, Cambridge, MA) for 3 days at 37 C under 5% CO_2. The cells were then pulsed with 0.5 μCi [^{3}H] thymidine (Amersham, Arlington Height, IL) for the last 6 h of incubation and harvested onto filter paper disks. Uptake of [^{3}H] thymidine was determined by scintillation counting, and the results were expressed as the stimulation index (S.I.), which was calculated as a ratio of the counts per minute in the presence or absence of antigen.

RNA extraction from PBMC, spleen cells, i-IEL and LPL

Isolated PBMC, splenocytes, i-IEL and LPL were adjusted to a concentration of 0.5 × 10^7 to 1.0 × 10^7/ml in RPMI 1640 supplemented with 10% FCS, 50 μg of streptomycin, 50 U of penicillin and 10 μg of gentamicin/ml, and then cultured with either 20 μg/ml of PPD or SIV Gag p27 at 37 C for 4 days. Non-stimulated cells were used as controls. Following culture, total cellular RNA was extracted according to the instructions provided with the RNeasy Mini Kit (QIAGEN, Valencia, CA) and stored at 80 C.

To investigate Gag-specific IFNγ responses in T cell subsets, $CD4^+$ and $CD8^+$ T cell populations from the immunized guinea pigs were obtained from PBMC and spleen cells using magnetic cell sorting (autoMACS) (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, after in vitro stimulation of total PBMC and spleen cells with 20 μg/ml of SIV Gag p27 at 37 C for 4 days, the cells were incubated with FITC-conjugated anti-guinea pig CD4 (Serotec Ltd., Oxford UK) or CD8 antibodies (Serotec Ltd.) followed by anti-FITC MicroBeads (Miltenyi Biotec). Non-stimulated cells were used as controls. $CD4^+$ T cell or $CD8^+$ T cell subpopulations

were negatively selected, and then total RNA was extracted with the RNeasy Mini Kit (QIAGEN).

Amplification of guinea pig IFNγ by reverse transcription (RT) and fluorogenic PCR

To examine antigen-specific IFNγ mRNA expression in PBMC, spleen cells, i-IEL and LPL, RT-PCR was performed using a TaqMan EZ RT-PCR kit according to the instructions provided (Applied Biosystems, Foster City, California). The reaction mixture consisted of 3 μl of appropriately diluted RNA sample; 5 μl of 5× TaqMan EZ buffer; 3 μl of 25 mM manganese acetate; 0.75 μl each of dATP, dCTP, dGTP and dUTP; 0.25 μl of primer for IFNγ at 100 μM; 1 μl of fluorogenic probe; 2.5 U of recombinant *Tth* DNA polymerase; 0.25 U of AmpErase uracil-*N*-glycosylase; and 8.25 μl of RNase-free water in a final volume of 25 μl. Thermal cycling conditions consisted of 2 min at 50°C, 30 min at 60°C and 5 min at 95 C, followed by 50 cycles of 10 s at 95°C and 45 s at 62°C. The ABI Prism 7700 sequence detection system (Perkin-Elmer, Applied Biosystems, Inc.) was employed for PCR cycling, real-time data collection and analysis.

Ribosomal RNA (rRNA) was used as an internal control, and its expression level was quantitatively determined using the TaqMan rRNA control reagent (Applied Biosystems) under the same conditions as described above.

The level of cytokine expression was determined in three independent samples for each animal. Quantification was normalized by dividing the amount of IFNγ mRNA in the target sample by the amount of rRNA in the same sample. Data are shown as fold induction of mRNA and expressed as the ratio of values obtained for antigen-stimulated cells to non-stimulated cells; ratios <1 indicate down-regulation, ratios >1 indicate up-regulation. Oligonucleotide primers (5′-CATGAACACCATCAAGGAACAAAT-3′, 5′-TTTGAATCAGGTTT-TTGAAAGCC-3′) and a fluorogenic-labeled probe (5′-6-carboxyfluorescein-TTCAAAGACAACAGCAGCAACAAGGTGC-6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine-3′) specific for guinea pig IFNγ mRNA were used for detection and quantification [30]. The RNA standard template used for quantitative determination of guinea pig-specific IFNγ mRNA was prepared as described by us elsewhere [30].

Antigen-specific serum antibody titration by ELISA

Sera were collected from each guinea pig at 50 weeks after immunization and stored at 80°C. Antigen-specific antibody titers were determined by ELISA. Maxisorp plates (Nunc A/S, Roskilde, Denmark) were coated with either PPD (0.5 μg/well) or SIV Gag p27 (0.1 μg/well) and incubated overnight at 4°C. Serially diluted sera were added to the wells and incubated for 2 h at 37°C. After three washes, rabbit anti-guinea pig IgG-horseradish peroxidase (HRP) conjugate (Zymed Laboratories, Inc., San Francisco, CA) was added to the wells and incubated for 2 h at 37°C, and the plates were then washed and visualized by adding TMB substrate (Moss, Inc., Pasadena, MD). After 30 min at room temperature, rates of absorbance were measured at 450 nm with an ELISA reader. Endpoint titers for antigen-specific IgG were calculated using Microsoft Office Excel and expressed as the last dilution giving an OD_{450} of 0.1 U above pre-immunization serum samples.

Statistical analysis

Data analysis was carried out with the Statistica program (StatSoft, Tulsa, OK). Data are expressed as the mean ± standard deviation (SD), and P values <0.05 were considered significant. DTH responses for the i.d. and orally immunized groups were compared using the unpaired t test, and the responses at week 8 and week 50 for each group were compared using the Student's paired t test. Levels of antigen-specific proliferative responses and IFNγ mRNA expression for the two groups were compared using the Mann Whitney U test because of variability in values among animals in each group.

Results

Construction of a rBCG-SIVGag vector expressing full-length SIV Gag

To achieve expression of the complete SIV Gag protein, we inserted the full-length *gag* DNA fragment of SIVmac239 into the plasmid pSO246, yielding a rBCG clone. The resulting recombinant clones (pSO246SIVGag and pSO246) were designated as rBCG-SIVGag and rBCG-pSO246, respectively. Transformation of cells with rBCG-SIVGag and analysis of the cell lysates by Western blot revealed a single band corresponding to 55 kDa consistent with the expected molecular weight of the SIV Gag protein (Fig. 1). The concentration of SIV Gag in the cell lysates was determined by SIV Gag p27 antigen ELISA and found to be 0.5 ng/1 mg of rBCG-SIVGag.

DTH skin responses to PPD and SIV Gag p27 antigen

For each vaccine, DTH skin tests for PPD and SIV Gag p27 antigens were performed at 8 and 50 weeks after immunization. DTH responses to PPD and Gag p27 antigens peaked 24 h after antigen injection. At week 8, DTH responses to PPD were detected in all six guinea pigs immunized i.d. with rBCG-SIVGag with a mean area of induration of 15.0 mm (Fig. 2A). The magnitude of induration in this group was similar to that seen in both the rBCG-pSO246- and BCG-Tokyo-inoculation groups (mean indurations = 15.5 and 15.0 mm, respectively). In contrast, only three of six animals orally immunized with rBCG-SIVGag exhibited a PPD-specific DTH response, and that response of six animals had a mean induration of 4.1 mm (Fig. 2A). Thus, it appeared that immunization with rBCG-SIVGag induced stronger DTH responses via the i.d. than the oral route (Fig. 2A). The three guinea pigs that showed no PPD-specific DTH responses were also included in further analyses of proliferation, levels of IFNγ mRNA expression and antibody production. Evaluation of animals receiving rBCG-pSO246 and BCG-Tokyo by the oral route also showed similar levels of PPD-specific DTH reactions (4.6 and 4.3 mm, respectively). At week 50, PPD-specific DTH responses were again detected in all six animals immunized i.d. with rBCG-SIVGag. The mean area of induration of these responses was 12.4 mm (Fig. 2A), equivalent to that seen in animals inoculated i.d. with either rBCG-pSO246 (11.0 mm) or BCG-Tokyo (13.5 mm). However, no DTH responses were seen in

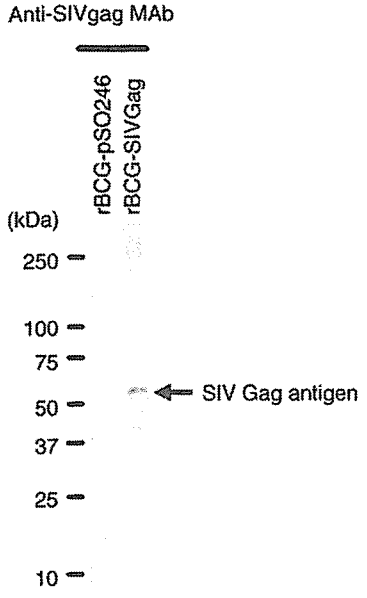

Figure 1 Western blot detection of SIV Gag in rBCG cell lysates. A 55-kDa molecule was identified, corresponding in size to the expected molecular weight of SIV Gag. Insertion of the empty plasmid 246 into rBCG (rBCG-pSO246) was used as a negative control.

any of the animals immunized orally with either rBCG-SIVGag, rBCG-pSO246 or BCG-Tokyo (Fig. 2A). With respect to DTH responses against SIV Gag p27, similar responses were seen at 8 weeks in groups i.d. and orally immunized with rBCG-SIVGag, with mean indurations of 15.8 and 16.1 mm, respectively (Fig. 2B).

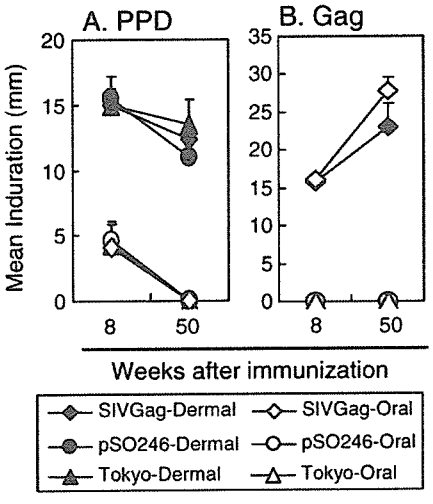

Figure 2 Induction of DTH skin reactions against (A) PPD and (B) SIV Gag p27 in guinea pigs intradermally or orally immunized with rBCG-SIVGag, BCG-Tokyo or rBCG-pSO246. DTH induction was performed at 8 and 50 weeks post-immunization. PPD (0.5 μg) and SIV Gag p27 (10 μg) were intradermally injected into the immunized animals, and the diameter of each area of induration was measured 24 h later. Only 3 of 6 animals orally immunized with rBCG-SIVGag exhibited PPD-specific DTH responses. Shown are the mean values ± standard deviation of all animals per group.

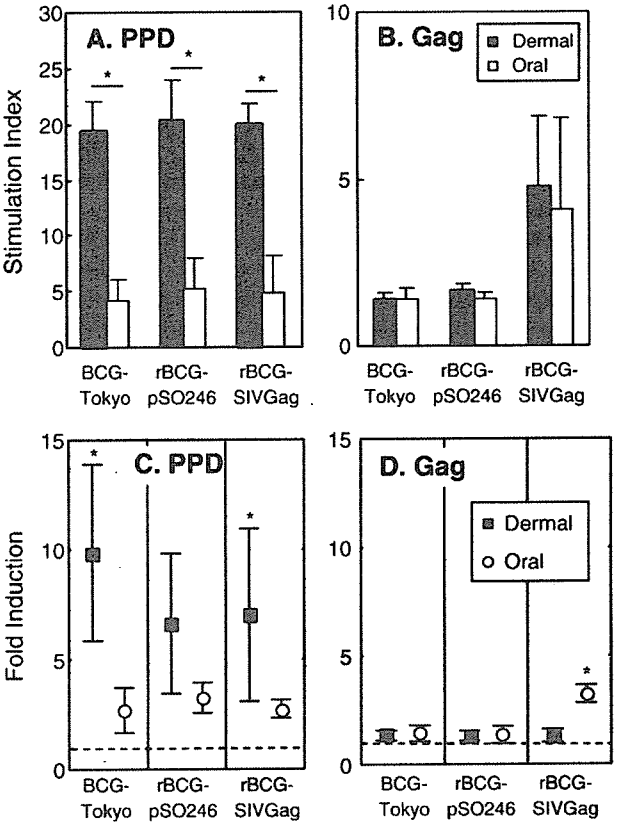

Figure 3 Induction of PPD- or SIV Gag-specific T cell proliferative responses (A, B) and IFNγ mRNA expression (C, D) in PBMC from guinea pigs intradermally or orally immunized with either rBCG-SIVGag, BCG-Tokyo or rBCG-pSO246. PBMC were isolated at week 20 after immunization and were cultured with or without antigen (20 μg/ml of either PPD or SIV Gag p27) for 3 days. During the final 6 h of incubation, 0.5 μCi [^{3}H] thymidine was added to each well. The cells were harvested, and the levels of [^{3}H] thymidine incorporation were determined by scintillation counting. Data are expressed as the stimulation index, as described in Subjects and methods. Shown are the means (solid bars, intradermal immunization; open bars, oral immunization) ± standard deviations. To investigate antigen-specific IFNγ responses, PBMC harvested 20 weeks after immunization were stimulated in vitro with antigen (20 μg/ml of either PPD or SIV Gag p27) for 4 days. Total RNA was extracted, and IFNγ mRNA levels were measured quantitatively by real-time RT-PCR. The results are expressed as the fold induction, as described in Subjects and methods. Shown are the mean values (symbols; ■, intradermal immunization; ○, oral immunization) ± standard deviations. *$P < 0.05$ (i.d. versus oral groups for each vaccine strain).

Interestingly, animals immunized with rBCG-SIVGag via either the i.d. or oral route showed strong, statistically identical SIV Gag p27-specific DTH responses (23.1 and 27.8 mm, respectively) at week 50 (Fig. 2B). The magnitude of the DTH to Gag at week 50 was significantly higher than that at week 8 in the group orally immunized with rBCG-SIVGag ($P = 0.004$), while it did not reach statistical significance in the i.d. immunized group. No significant levels of Gag p27 antigen-specific DTH responses were detected in animals inoculated with either rBCG-pSO246 or BCG-Tokyo via either the i.d. or oral routes (Fig. 2B). In addition, no significant

DTH responses to PPD and Gag p27 antigens were found in control animals inoculated with saline alone (data not shown).

Antigen-specific proliferative responses and IFNγ mRNA expression in PBMC

To investigate the T cell responses specific for SIV Gag p27 and PPD antigens, proliferation assays were performed at week 20 after immunization (Figs. 3A and B). In the groups immunized with rBCG-SIVGag, PPD-specific proliferative responses were evaluated in PBMC from the immunized animals. The levels of these responses were found to be significantly higher in the i.d. immunized group than in the orally immunized group (19.9 ± 2.0 and 4.9 ± 3.2, respectively, $P = 0.02$) (Fig. 3A). Conversely, similar levels of proliferative responses to SIV Gag p27 were elicited by either i.d. or oral immunization with rBCG-SIVGag (4.8 ± 2.1 and 4.1 ± 2.8, respectively) (Fig. 3B). PPD-specific proliferative responses to BCG-Tokyo or rBCG-pSO246 were 19.3 ± 2.8 and 20.2 ± 3.9, respectively, in the i.d. group, while those inoculated by the oral route had proliferative responses of 4.2 ± 1.9 and 5.3 ± 2.6, respectively.

To assess Th1-type helper T cell functions specific for PPD and SIV Gag, IFNγ mRNA expression in PBMC was measured quantitatively at week 20 using real-time, fluorogenic RT-PCR (Figs. 3C and D). In animals receiving rBCG-SIVGag i.d. or orally, the level of IFNγ mRNA expression was elevated following stimulation with PPD over baseline values obtained from non-stimulated PBMC. Mean values specific for PPD in the i.d. and orally immunized groups were 7.0 ± 3.9 and 2.8 ± 0.4, respectively ($P = 0.04$, Fig. 3C). With respect to IFNγ mRNA expression specific for SIV Gag p27, the level was enhanced in animals immunized orally with rBCG-SIVGag, whereas not activated in animals vaccinated i.d. with the vaccine (3.3 ± 0.4 and 1.3 ± 0.3, respectively, $P = 0.02$, Fig. 3D). By comparison, the levels of PPD-specific IFNγ mRNA expression were 9.9 ± 4.0 and 6.8 ± 3.2, respectively, in animals receiving either BCG-Tokyo or rBCG-pSO246 by the i.d. route, and 2.7 ± 1.0 and 3.2 ± 0.7, respectively, for animals inoculated by the oral route.

Long-term antigen-specific IFNγ mRNA expression in PBMC, spleen cells, i-IEL and LPL

To assess whether Th1-type T cells persist in systemic and mucosal compartments, the level of IFNγ mRNA expression was determined at week 50 using PBMC, splenocytes, i-IEL and LPL from guinea pigs immunized either i.d. or orally with rBCG-SIVGag (Fig. 4). Higher levels of IFNγ mRNA induced by PPD were clearly detected in PBMC from guinea pigs immunized with rBCG-SIVGag (Fig. 4A). However, the mean levels of PPD-specific IFNγ mRNA expression were significantly higher in animals immunized by the i.d. route compared to the oral route (205.0 ± 51.2 and 15.5 ± 19.4, respectively, $P = 0.02$) (Fig. 4A). Similarly, the levels of Gag p27-specific IFNγ mRNA expression in the rBCG-SIVGag immunized animals were higher in the i.d. group compared with those in the orally immunized group, but these differences did not reach significance (53.3 ± 50.9 and 6.4 ± 3.2, respectively, $P = 0.15$, Fig. 4B). PBMC from control animals inoculated with either BCG-Tokyo or rBCG-pSO246 had levels of PPD-specific IFNγ mRNA comparable to animals vaccinated with rBCG-SIVGag via same immunization route (Fig. 4A). As expected, no Gag-specific IFNγ mRNA responses were found in animals inoculated with either BCG-Tokyo or rBCG-pSO246 (Fig. 4B).

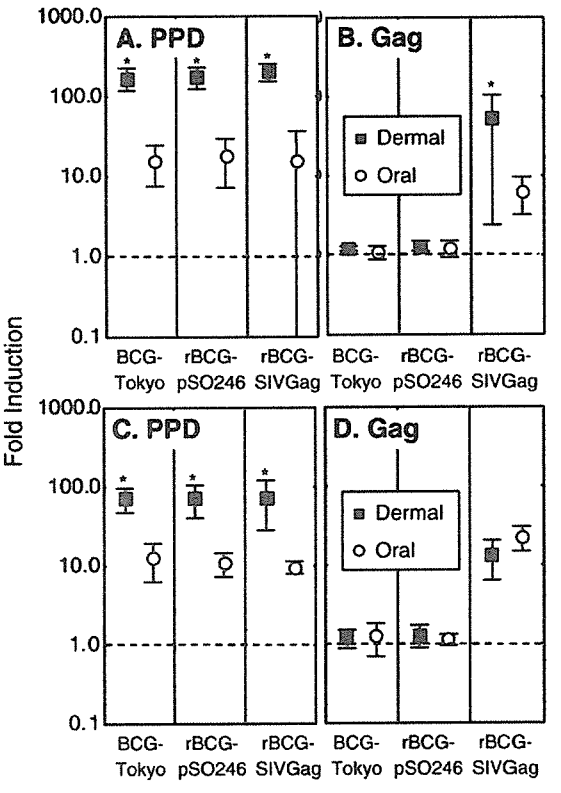

Figure 4 Profile of IFNγ responses at 50 weeks against PPD and SIV Gag p27 antigens in PBMC (A, B) and spleen cells (C, D) from guinea pigs intradermally or orally immunized with rBCG-SIVGag, BCG-Tokyo or rBCG-pSO246. Cells harvested 50 weeks after immunization were stimulated in vitro with antigen (20 μg/ml of either PPD or SIV Gag p27) for 4 days. Total RNA was extracted, and IFNγ mRNA levels were measured quantitatively by real-time RT-PCR. The results are expressed as the fold induction. Shown are the mean values (symbols; ■, intradermal immunization; ○, oral immunization) ± standard deviations. *$P < 0.05$ (i.d. versus oral groups for each vaccine strain).

Splenocytes from guinea pigs receiving rBCG-SIVGag expressed considerably higher levels of IFNγ mRNA in response to PPD and SIV Gag p27 compared to baseline values obtained from non-stimulated splenocytes (Figs. 4C and D). PPD-specific IFNγ responses were significantly higher in animals immunized with rBCG-SIVGag by the i.d. route (75.0 ± 46.5) compared with those immunized by the oral route (9.7 ± 1.7, $P = 0.02$, Fig. 4C). Marked increases in the response to SIV Gag p27 were found in both i.d. and orally immunized animals receiving rBCG-SIVGag (13.6 ± 7.2 and 22.8 ± 7.8, respectively, Fig. 4D). Splenocytes from animals inoculated with either BCG-Tokyo or rBCG-pSO246 had levels of PPD-specific IFNγ responses similar to those from animals vaccinated with rBCG-SIVGag via the same immunization route (Fig. 4C), while no Gag-specific IFNγ responses were observed in these animals (Fig. 4D).

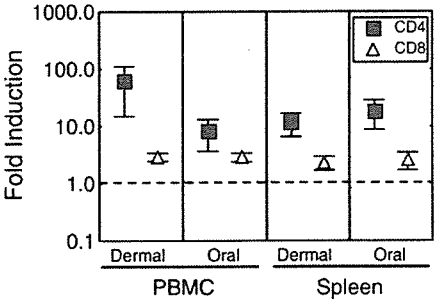

Figure 5 Profile of IFN-γ responses at 50 weeks against SIV Gag p27 antigen in PBMC and spleen cells from guinea pigs intradermally or orally immunized with rBCG-SIVGag. Cells harvested 50 weeks after immunization were stimulated in vitro with antigen (20 μg/ml of SIV Gag p27) for 4 days. After separation of $CD4^+$ and $CD8^+$ T cell subsets, total RNA was extracted, and IFN-γ mRNA levels were measured quantitatively by real-time RT-PCR. The results are expressed as the fold induction. Shown are the mean values (symbols; ■, $CD4^+$ T cell subsets; △, $CD8^+$ T cell subsets) ± standard deviations.

To further investigate Gag-specific IFN-γ responses in T cells, PBMC and splenocytes from guinea pigs vaccinated with rBCG-SIVGag were separated at week 50 into $CD4^+$ and $CD8^+$ T cell subsets. Higher levels of IFN-γ mRNA expression were detected in $CD4^+$ T cells in comparison to $CD8^+$ T cells from PBMC and splenocytes from guinea pigs in both the i.d. and oral immunization groups (Fig. 5). The mean values of Gag-specific IFN-γ responses were 63.5 ± 48.4 and 8.4 ± 4.8 for PBMC $CD4^+$ T cells, and 11.7 ± 5.2 and 18.8 ± 10.0 for spleen-derived $CD4^+$ T cells, in the i.d. and orally immunized groups, respectively. Comparatively, the magnitude of Gag-specific IFN-γ responses in $CD8^+$ T cell subsets from PBMC and splenocytes ranged from 2.3 to 3.0 in the i.d. and orally immunized animals (Fig. 5). These results indicate that long-lasting, Gag-specific IFN-γ responses are induced by immunization with rBCG-SIVGag, and these responses are mediated to a large extent by $CD4^+$ T cells at 50 weeks after vaccination.

PPD- and Gag-specific IFN-γ responses were also determined for i-IEL from the large intestines of guinea pigs receiving rBCG-SIVGag. The mean values of PPD-specific IFN-γ responses were 1.1 ± 0.4 and 0.7 ± 0.1 for the i.d. and oral groups respectively, while those for Gag-specific IFN-γ responses were 1.7 ± 0.7 and 0.8 ± 0.1, respectively. Similarly, LPL from the small intestines of the i.d. and orally immunized animals had mean values for PPD-specific IFN-γ responses of 1.0 ± 0.2 and 1.1 ± 1.0, respectively, while the means for Gag-specific IFN-γ responses were 1.4 ± 0.2 and 1.3 ± 0.7, respectively. LPL from the large intestines exhibited no significant increase in antigen-specific IFN-γ mRNA expression. i-IEL from the small intestines were not isolated in sufficient quantity for analysis due to the enormous quantity of mucus, which is copious in the small intestine. Thus, our results indicate that no significant increases occurred in levels of IFN-γ mRNA expression upon in vitro stimulation with PPD or SIV Gag p27 in i-IEL and LPL from guinea pigs immunized with rBCG-SIVGag by either route. Previous studies have suggested that i-IEL and LPL have different activation requirements than do PBMC [31,32]. Further study is needed on the antigen-specific mucosal immunity induced by rBCG-SIVGag.

Induction of antigen-specific serum antibody responses

To investigate the induction of humoral immune responses to PPD and SIV Gag p27 in guinea pigs immunized with rBCG-SIVGag, antigen-specific serum IgG titers were determined by ELISA (Fig. 6). Even at 50 weeks after immunization,

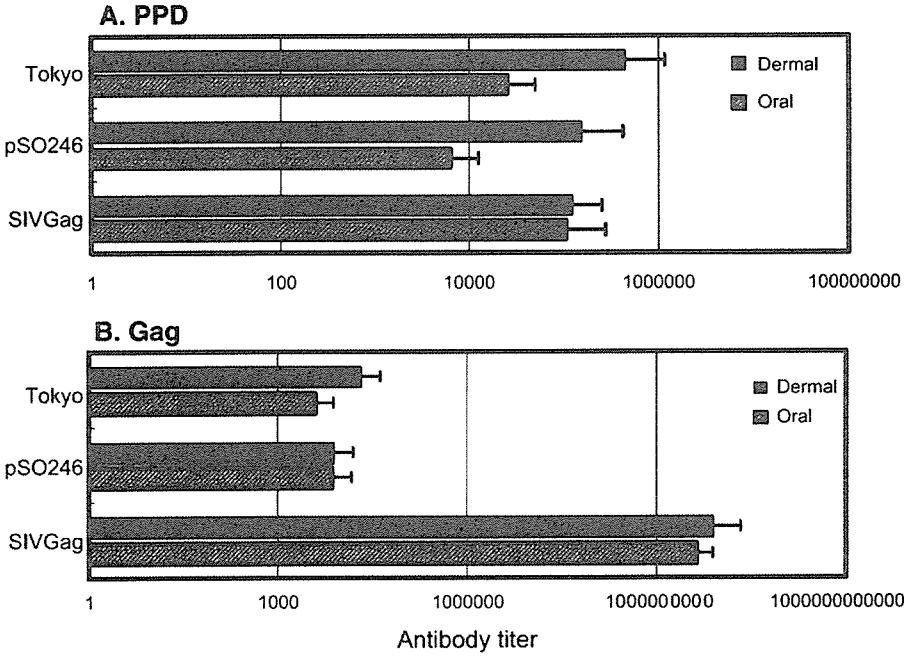

Figure 6 Induction of serum IgG responses against (A) PPD and (B) SIV Gag p27 antigens in guinea pigs intradermally (solid bars) or orally (hatched bars) immunized with either BCG-Tokyo, rBCG-pSO246 or rBCG-SIVGag 50 weeks after immunization. Shown are the mean values ± standard deviations.

significant levels of serum IgG specific for PPD and SIV Gag p27 were detected in all animals from both immunization groups. In comparison to animals inoculated with control preparations of BCG-Tokyo or rBCG-pSO246, the levels of Gag-specific serum IgG in the animals receiving rBCG-SIVGag were 10^6-fold higher, although low but detectable levels of non-specific IgG against Gag p27 antigen could be found in the animals receiving control inoculations compared to non-immunized healthy animals (Fig. 6B). As expected, animals inoculated with BCG-Tokyo and rBCG-pSO246 had serum IgG titers against PPD similar to those seen in the rBCG-SIVGag-immunized group (Fig. 6A).

Discussion

We report here that i.d. or oral inoculation with rBCG-SIVGag induces SIV- and tuberculosis-specific immune responses in guinea pigs, and these responses persist for at least 1 year (the duration of the study period). These findings suggest that rBCG-Gag might be used as an immunogen to elicit responses specific for HIV as well as tuberculosis at human doses.

In this study, we used BCG-Tokyo as a parental strain to develop a rBCG vector and confirmed that both the parental BCG and the rBCG had the same advantage of providing long-lasting, cell-mediated, PPD-specific immunity after a single i.d. inoculation. Furthermore, two oral inoculations of rBCG also produced levels of immunity against PPD identical to those seen with the parental BCG. We have evidence that both i.d. and oral inoculation of rBCG can elicit positive immunity against challenge by inhaled *M. tuberculosis* with similar efficacy to that conferred by vaccination with BCG-Tokyo [S. Haga et al., personal communication]. Thus, the ability to induce antigen-specific immunity and provide protective efficacy against *M. tuberculosis* may apply to rBCG as well as the BCG-Tokyo vaccine strain.

In studying the rBCG, we sought to determine how effectively BCG might express an inserted gene from a foreign immune deficiency virus. We found that rBCG produced intracellular SIV Gag protein at a level of 0.5 ng/mg. Even though i.d. inoculation dosages were small, 0.1 mg of rBCG-SIVGag and 0.05 ng of SIV Gag protein per animal, we achieved the induction of a strong immune response specific for SIV Gag that was maintained over a 50-week period of observation. Several possible attributes may contribute to the ability of rBCG to elicit potent immune responses: (i) the BCG bacillus is known to increase immune responses by acting as an adjuvant; (ii) the persistence of live rBCG in host cells may account for its ability to induce long-lasting, specific immunity; and (iii) secretory proteins derived from the mycobacteria, such as α-antigen (also known as MPB59 or antigen 85B), can elicit potent Th1 immune responses [33 35], which have been shown to be beneficial for controlling pathogenic infectious agents [36 39].

The rBCG-SIVGag used here was able to effectively elicit long-term, SIV Gag-specific DTH, proliferative and IFNγ responses in PBMC and splenocytes from either i.d. or orally immunized guinea pigs. Previous reports have shown that the degree of HIV-specific DTH responsiveness, which generally depends on the intensity of helper T cell function, correlates with clinical stability in infected individuals [40,41]. Furthermore, several lines of evidence support the importance of maintaining helper T cell function in controlling viral infection and replication [42,43]. A related study has suggested that the maintenance of HIV-1 Gag-specific proliferative responses helps preserve Gag-specific CTL activity [44]. In addition, IFNγ has been shown to play an important role in controlling HIV-1 and SIV replication [45 48] and *M. tuberculosis* infection [49 51]. In a recent study of HIV-1-infected subjects, the production of IFNγ in response to Gag was associated with a lower viral load set point [52]. Based on these observations, it is conceivable that immunization with rBCG-SIVGag might help control viral load and curb disease progression, although this has yet to be tested in the appropriate animal models.

In the present study, the levels of IFNγ mRNA induced in response to both PPD and Gag were elevated at week 50 compared to week 20. The cells were re-stimulated in vitro with the respective antigens; therefore, our results reflect the IFNγ reaction of effector cells that were differentiated from memory T cells and activated by in vitro restimulation. This finding might be attributed to the number of memory T cells or the ability of the memory cells to differentiate into effector cells. The amount of rBCG-produced Gag antigen is very low, perhaps resulting in low levels of Gag-specific IFNγ responses at week 20. However, once the Gag-specific IFNγ response was evoked by rBCG-Gag, the response was maintained for more than 1 year after immunization. As shown in Fig. 6, serum IgG against PPD and Gag was detected at high levels even at week 50 in rBCG-Gag-immunized guinea pigs, suggesting persistent antigenic stimulation by chronic rBCG infection. Such chronic infection might gradually lead to an increase in memory T cell counts and/or enhancement of differentiation into effector cells.

In guinea pigs, IFNγ-specific tetramers, ELISPOT assays and flow cytometric analyses remain to be developed. Hence, we relied on a method for quantitative determination of antigen-specific IFNγ mRNA expression in $CD4^+$ and $CD8^+$ T cell subpopulations using real-time RT-PCR. Our results indicate that long-lasting IFNγ responses against SIV Gag p27 induced by rBCG-SIVGag inoculation occur mainly in the $CD4^+$ T cell population and not the $CD8^+$ T cell population at the 50-week time point. However, it is unclear whether a $CD8^+$ T cell IFNγ response to Gag p27 may have occurred because the level of Gag-specific IFNγ expression was normalized by using the amount of rRNA in the sample. Namely, in case of a low frequency of Gag-specific memory $CD8^+$ T cells in the sample, it may be difficult to accurately detect enhancement of the Gag-specific IFNγ expression even if such memory $CD8^+$ T cells are activated by restimulation with Gag antigen.

The current study demonstrates that i.d. immunization with rBCG-SIVGag or BCG-Tokyo induces significantly higher DTH responses to PPD than does oral immunization with the same vaccines. Interestingly, guinea pigs lacking a PPD-specific DTH response 8 weeks after oral inoculation with rBCG-SIVGag still exhibited significant proliferative and IFNγ responses to the PPD antigen. Moreover, a significant IFNγ response to PPD was generated in guinea pigs in which a PPD-specific DTH reaction was no longer detected at week 50. These results clearly indicate that a DTH reaction to PPD does not necessarily reflect proliferative and IFNγ responses [30].

It is possible that a T cell subset mediating a DTH response to PPD [53] was transiently localized at a cutaneous area by oral inoculation with rBCG-SIVGag. However, oral immunization with rBCG-SIVGag effectively induced Gag-specific DTH responses over a 50-week period. These findings indicate that oral inoculation of guinea pigs with rBCG-SIVGag engenders distinct DTH kinetics between PPD and Gag antigens, suggesting that different T cell subsets may be responsible. It has been shown that T cells activated by oral vaccination of humans with common BCG preferentially express a mucosal homing $\alpha 4\beta 7$ molecule associated with T cell trafficking to mucosa, resulting in a failure of the immunization regimen to induce PPD-specific DTH responses [54]. However, in this study of guinea pigs, it is unclear why oral rBCG-SIVGag inoculation persistently elicited vigorous Gag-specific DTH responses. It is possible that intracellular expression of SIV Gag within BCG inoculated orally may not be efficiently recognized by antigen-presenting cells in gut-associated lymphoid tissue (GALT). Namely, SIV Gag antigens might be processed and presented after migration of rBCG-SIVGag-infected macrophages to systemic compartments (e.g., the spleen). In the oral immunization group, a Gag-specific T cell subset may then home to systemic compartments including a cutaneous area, rather than to the mucosa. It will be important in future studies to investigate whether mucosal and cutaneous homing molecules are expressed on PPD- and Gag-specific T cell subsets that are induced by oral inoculation with rBCG-SIVGag.

Intradermal or oral vaccination of guinea pigs with rBCG-SIVGag resulted in the production of serum IgG directed to SIV Gag p27 and PPD. Although it is unclear how Gag-specific IgG affects HIV-1 infection or replication in vivo, a reduction in anti-Gag antibody levels has been shown to correlate with the onset of disease progression [55,56]. More recently, it has been reported that IgG2 directed against Gag is associated with a low viral load and high levels of antigen-specific IFNγ production [57]. We were unable to determine the IgG subclass of the serum antibodies in the immunized guinea pigs because of the lack of species-specific reagents. However, it is possible that the Gag-specific IgG observed here might be classified into a subclass corresponding to IgG2 of humans, since a significant Gag-specific IFNγ response was detected. In addition, we previously found that nasal immunization of mice with rBCG Env V3 induced not only antigen-specific IFNγ but higher levels of V3 antigen-specific serum IgG2 than IgG1 [58].

Thus, we have shown that a rBCG vaccine can induce antigen-specific immunity to viral as well as bacterial antigens. It is especially interesting to note that significant levels of Gag-specific immunity were induced by inoculation with rBCG-SIVGag at the dose and route commonly used for BCG vaccination in humans (0.1 mg by i.d. inoculation). These findings suggest that a rBCG-based vaccine targeting the HIV-1 Gag region might be an effective immunogen. Currently, many candidate HIV-1 vaccines are multivalent, utilizing several viral proteins for the induction of broadly reactive virus-specific immune responses. However, recent studies have shown the effectiveness of SIV vaccines expressing a single viral Gag protein, including Mamu-A*01 macaques immunized with either SIV Gag DNA [59] or adenovirus type 5 vectors expressing SIV Gag proteins [60]. Results using these vaccines indicate that expression of Gag alone is sufficient to induce significant efficacy in the macaque model.

However, there are certain drawbacks to using rBCG as a live, vector-based vaccine. One of the most serious concerns is that it might interfere with immunity induced by other tuberculosis vaccines that are based on the same BCG vector. Pre-existing immunity in BCG-vaccinated individuals may lead to rapid neutralization of a rBCG vaccine. However, the anamnestic effect of BCG vaccination would be irrelevant if our ultimate goal is reached that is, the replacement of common BCG vaccination by administration of a rBCG vaccine to newborns who have no pre-existing immunity to BCG. While it is clear that the safety of a rBCG vaccine must be established for use in humans, BCG-Tokyo may be one of the most suitable BCG substrains to use as an HIV vaccine vector because it is less virulent than other substrains and its inoculation does not cause severe systemic infection in immune deficient animals [61 64]. Based on these findings, rBCG based on BCG-Tokyo may have promise as a suitable vector for an HIV/AIDS vaccine.

Although the current study did not directly address the efficacy of the rBCG-SIVGag vaccine against viral challenge due to the failure of guinea pigs to support infection with HIV or SIV, our results open up the possibility of i.d. immunization with a single, human dose of rBCG-HIVGag against both HIV and tuberculosis; an immunization regimen that might one day replace the common BCG vaccine without requiring any variation in the current dose or protocol.

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Research paper

Novel two-parameter flow cytometry (MIL4/SSC followed by MIL4/CT7) allows for identification of five fractions of guinea pig leukocytes in peripheral blood and lymphoid organs

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Abstract

Though the guinea pig has been an extremely useful animal model for a variety of diseases, the tools necessary to undertake a full-scale immunological analysis of the guinea pig have been lacking. For instance, traditional two-parameter forward/side scatter (FSC/SSC) flow cytometry, though effective in human and other animal models, is unable to adequately identify the distinct fractions of guinea pig peripheral blood leukocytes (PBL). We introduce here a new flow cytometric technique (MIL4/SSC followed by MIL4/CT7) which redresses this lack by identifying and characterizing five distinct fractions of PBL: neutrophils, lymphocytes, monocytes, eosinophils plus basophils, and the novel MIL4^{-}SSC^{large}CT7^{high} population. The MIL4^{-}SSC^{large}CT7^{high} cells possess cytoplasmic inclusion bodies of variable size that were positive for periodic acid Schiff (PAS). Their cell surface stained positive for the helper/inducer lymphocyte markers, T cell markers, CD45, Thy-1, asialo GM1 and FcR, but negative for B cell markers, such as membrane-type IgM, CD8 and MHC class II. The novel flow cytometric technique also allowed us to establish that the five leukocyte fractions were found in PBL, splenocytes, thymocytes and lymph node cells. Cells which were positive for inclusion bodies comprised 16.6% of splenocytes, 9.9% of PBL and 4.3% of liver cells, but were comparatively rare in lymph node cells, thymocytes, and BM cells. The novel flow cytometric technique introduced here will allow a better understanding of the response of each type of guinea pig leukocyte and thereby shed light on the diseases with which they are associated.

Keywords: Guinea pig; Leukocyte fraction; Flow cytometry; Cell surface marker

Abbreviations: BCG, bacillus Calmette and Guérin; MHC, major histocompatibility complex; PBL, peripheral blood leukocytes; CTL, cytotoxic T lymphocyte; MIL4^{-}SSC^{large}CT7^{high}, MIL4^{-}side scatter^{large}CT7^{high}; NIID, National Institute of Infectious Diseases; FITC, fluorescein isothiocyanate; PE, phycoerythrin; mAb, monoclonal antibody; APC, allophycocyanin; LN, lymph node; BM, bone marrow; PI, propidium iodide; PAS, periodic acid Schiff; PMN, polymorphonuclear neutrophils.

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1. Introduction

For more than 100 years, guinea pigs have been an important animal model for the study of *Mycobacterium tuberculosis* (*M. tuberculosis*), the vaccine strain *M. bovis* bacillus Calmette and Guérin (BCG) (Bloom and Fine, 1994) and other infectious agents (Griffith and Aquino-de Jesus, 1991; Myers and Connelly, 1992; Stanberry, 1995; Wicher and Wicher, 2001; Woolf, 1991); carcinoma (Van der Meijden et al., 1989); allergies (Broder et al., 1978; Daffonchio et al., 1989; Gulbenkian et al., 1990; Kallos and Kallos, 1984; Samejima et al., 1988; Tohda et al., 2001); and various other biological phenomena (Wagner and Manning, 1976).

Though an animal model of considerable importance, guinea pigs pose some immunological limitations not seen in other models. For instance, in humans PBL leukocyte fractions of granulocytic, lymphoid or mononuclear phagocytic origins are easily identified (Abramson et al., 1977; Prchal et al., 1978) using traditional flow cytometry, and terminally differentiated cells within those fractions are known to function cooperatively as a major defense against foreign pathogens (Ginaldi et al., 1999; Silva et al., 1989; Paul, 1993, 1999; Zhang et al., 1992). However, those fractions cannot be clearly identified in guinea pigs using standard two-parameter forward/side scatter (FSC/SSC) flow cytometric analysis (Choong et al., 1995; Goto and Nishioka, 1989; Lanza et al., 1992).

Other obstacles to the identification of distinct PBL populations in guinea pigs include the lack of phenotypic characterization of cell surface markers due to the limited availability of anti-guinea pig antibodies (only ten clones are available), the paucity of genetic data on guinea pig MHC haplotypes, the lack of well-established animal strains, and the lack of data on the functional properties of guinea pig leukocytes, for example cytotoxic T lymphocyte (CTL) activity. Finally, guinea pig spleen cells do not always respond well to immunogens, underscoring the challenge of using these animals in immunologic studies.

To help redress these problems, we found cross-reactivity of porcine m-Ab MIL4 (Haverson et al., 1994) and here introduce a new flow cytometric technique (MIL4/SSC followed by MIL4/CT7 antibodies) capable of clearly differentiating five distinct fractions of guinea pig leukocytes: neutrophils; monocytes; lymphocytes; eosinophils plus basophils; and the novel MIL4^{-}side scatter^{large}CT7^{high} (MIL4^{-}SSC^{large}CT7^{high}) leukocytes. We also used this technique to characterize the fractions and to normalize tissue distribution.

2. Materials and methods

2.1. Animals

Female Hartley guinea pigs, 4 to 6 weeks of age and weighing 350 to 400 g each, were obtained from Japan SLC, Inc., Shizuoka, Japan Animals used in the studies were free of pathogenic bacteria and parasites. They were housed in accordance with the guidelines for animal experimentation of the Japanese Association for Laboratory Animal Science, 1987, in full compliance with the Law for the Humane Treatment and Management of Animals (in Japan). Animals were fed and maintained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of National Institute of Infectious Diseases (NIID), Japan. Once approved by an institutional committee for animal experiments, these studies were conducted at the Animal Facility of Toyama Campus, NIID, Japan, in accordance with the requirements specifically stated in the Laboratory Biosafety Manual of the World Health Organization.

2.2. Antibodies

The anti-guinea pig antibodies used in this study are listed in Table 1. Fluorescein isothiocyanate (FITC)-

Table 1
Panel of anti-guinea pig mAbs used for flow cytometry[a]

Clone	Specificity	Ig class
Msgp9	Pan B-cell	IgG1
31D2	IgM	IgG
R27E	MHC Class II	IgG
20ED7	FcR	IgG
CT5	Pan T-cell	IgG1
H159	Pan T-cell	IgG
CT7	CD4 (T helper/inducer)	IgG1
H155	T helper/inducer	IgG
H154	Thy-1	IgG
CT6	CD8 (T suppressor/cytotoxic)	IgG1
MIL4	Porcine neutrophils and eosinophils	IgG1
Asialo GM1	Mouse and rat NK cells	Polyclonal rabbit Ig
IH-1	LCA (leukocyte common antigen)	IgG1
H201	LCA (leukocyte common antigen)	IgG

[a] Anti-guinea pig mAbs R27E, 31D2, 20ED7, H159, H155, H154 and H201 were the kind gift of Dr. R. Burger, Robert Koch Institute, Berlin, Germany. Asialo GM1 was obtained from Wako Pure Chemicals, Ltd., with the rest obtained from Serotec.

labeled T helper/inducer (CT7) cells, anti-CD8 cells (CT6, Samejima et al., 1988), anti-B cells (Msgp9) and anti-T cells (CT5, Broder et al., 1978), along with the purified anti-porcine neutrophil subset (MIL4, Haverson et al., 1994), anti-CD45 (IH-1) and phycoerythrin (PE)-labeled rabbit anti-mouse IgG-F(ab')$_2$ were obtained from Serotec, Ltd. (Oxford, UK). The MIL4 monoclonal antibody (mAb) was labeled with FITC and biotin by conventional methods. Streptavidin–allophycocyanin (APC) and streptavidin–cychrome was purchased from Pharmingen (San Diego, CA, USA). Antibodies to guinea pig MHC class II antigens (R27E7), IgM (31D2), leukocyte common antigen (LCA, H201), an additional T cell antigen (H159), an additional T helper/inducer antigen (H155), Thy-1 (H154) and Fc receptors (20ED7) were kindly supplied by Dr. R. Burger, Department of Immunology, Robert Koch-Institute, Nordufer 20, D-13353, Berlin, Germany (Burger et al., 1981; Schafer and Burger, 1991; Steerenberg et al., 1991). Anti-guinea pig Fc receptor (20ED7) was prepared by one of us (Chiba, unpublished). Anti-asialo GM1 was obtained from Wako Pure Chemicals, Ltd. (Tokyo, Japan). Anti-human CD56 antibodies used in the study were NCAM16.2 (Becton Dickinson, San Jose, CA, USA), MY 31 (Becton Dickinson), B159 (Pharmingen), NKI-nbi-1, (Nichirei Bioscience Inc., Tokyo, Japan) and NKH-1 (Beckman Coulter, Florida, USA).

2.3. Cells and isolation of leukocyte subsets

Splenocytes, lymph node (LN) cells, thymocytes and liver cells were teased out by pressing organ fragments through a mesh filter cell strainer (Becton Dickinson). PBL, liver cells, bone marrow (BM) cells and splenocytes were hemolysed using a lysis buffer consisting of 0.15M NH_4Cl, 0.1mM EDTA-2Na, 1.0mM $KHCO_3$. After being washed twice, the cells were resuspended in PBS-staining buffer with 0.1% NaN_3 and 3% FCS.

2.4. Flow cytometric analysis of PBL and immune cells by their cell-surface staining with specific monoclonal antibodies

First, normal guinea pig γ-globulin was purified by 33% by subjecting it to two courses of ammonium sulfate precipitation and then incubated with cells at a final concentration of 1mg/ml at 4°C for 10min to prevent nonspecific binding of the specific antibodies, except for asialo GM1 (Nakahara et al., 1980), FcR, and IgM staining. Then, we added a purified antibody and incubated for a further 30min before washing and adding a PE-conjugated rabbit anti-mouse IgG F (ab) at 4°C for 30min. FITC-conjugated and biotin-conjugated antibodies were incubated with the cells at 4°C for 30min. Finally, we incubated the cells with streptavidin APC and streptavidin cychrome at 4°C for 30min and washed with staining buffer. Dead cells were excluded by propidium iodide (PI) and viable cells were analyzed on a FACScalibur (Becton-Dickinson, San Jose, CA, USA) equipped with an argon and semiconductor laser sets at 488 and 635nm using Cell Quest software (Becton-Dickinson).

2.5. Morphological studies

Sorted cells were cytocentrifuged using a Cytospin-3 (Shandon Life Sciences International, Ltd., Runcorn, UK) and stained with May–Giemsa solution (Merck KGaA, Darmstadt, Germany) or periodic acid Schiff (PAS) (MUTO Pure Chemicals Co., Ltd., Tokyo, Japan), as described elsewhere.

2.6. Statistical analysis

Data analysis was carried out using the Stat View program (SAS Institute, Cary, NC) and data are expressed as the mean±SD.

3. Results

3.1. Evaluation of guinea pig leukocytes by conventional flow cytometry

As noted above, one of the drawbacks of studying immunologic responses in guinea pigs is the difficulty of identifying distinct PBL populations by conventional assay methods (guinea pig PBL, 1A-a; and human PBL, 1B-a). We found that one reason for the difficulty in gating guinea pig PBL was a unique population of cells in guinea pigs (the gated cell population in 1A-b), not present in humans (no corresponding cells in the gated area in Fig. 1B-b), that stains highly positive for the helper/inducer marker CT7 and SSC (the gated cell population in Fig. 1A-b). When the $SSC^{large}CT7^{high}$ population was deleted from guinea pig PBL in the cytometric analysis data, the three distinct populations of lymphocytes, gated with green circles; monocytes, gated with red circles; and granulocytes, gated with blue circles, could be clearly visualized using the conventional FSC/SSC flow cytometry of guinea pig PBL (Fig. 1A-c).

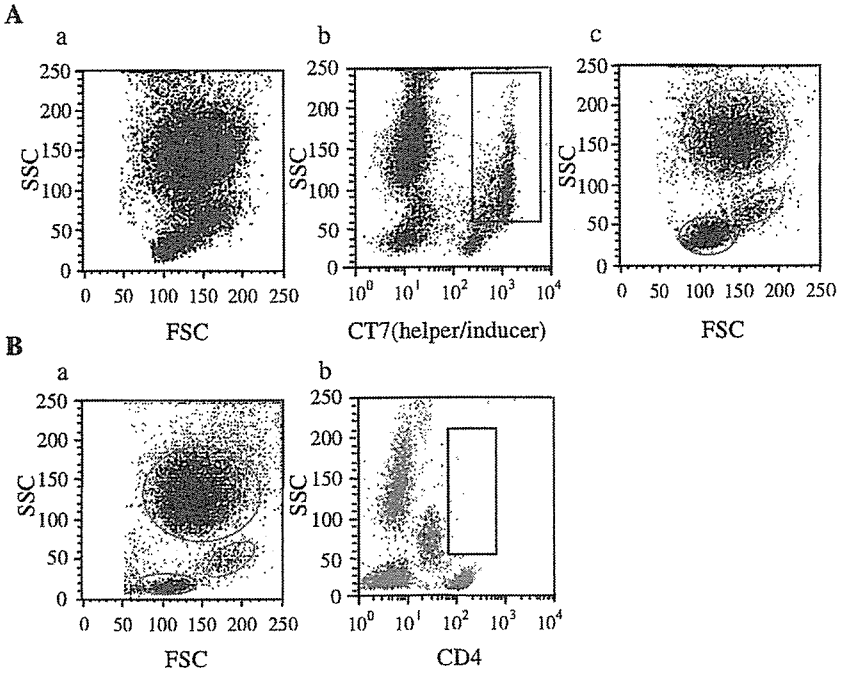

Fig. 1. Comparison of guinea pig and human PBL by two-parameter flow cytometric assays using light scatter and anti-CT7 (helper/inducer) mAb. Viable cells were gated by FSC/SSC after excluding dead cells using propidium iodide. A. Separation of guinea pig PBL fractions: (a) unsuccessful separation of guinea pig PBL by conventional FSC/SSC two-parameter flow cytometry. (b) Another separation of the PBL by CT7/SSC two-parameter flow cytometry. (c) Distribution of PBL after removal of the $SSC^{high}CT7^{high}$ population which was located in the gated population of Fig. 1A-b. B. Separation of human PBL fractions. Two-parameter flow cytometric separation of PBL by (a) FSC/SSC and (b) CD4/SSC. Each gated color population from humans was equivalent to the color indicated by SSC and CD4: lymphocytes (green), monocytes (red), and granulocytes (blue). Gate in Fig. 1B-b contained a SSC^{high}CD4-positive population.

3.2. The unique PBL fraction is further identified as $MIL4^{-}SSC^{large}CT7^{high}$

By screening human and mouse mAbs that cross-react with guinea pig cell surface antigens, we identified one antibody MIL4 that proved useful in differentiating guinea pig PBL into four subpopulations using two-parameter, consecutive MIL4/SSC flow cytometry (Fig. 2A). That antibody had previously been shown to recognize porcine neutrophils, eosinophils and basophils (Haverson et al., 1994). However, the CD antigen which was recognized by this antibody has not been defined (Haverson et al., 1994). By comparing the flow charts of guinea pig PBL obtained using a two-parameter MIL4/SSC analysis (Fig. 2A) with that obtained using a FSC/SSC analysis (Fig. 2B), we were able to identify four distinct PBL subpopulations: lymphocytes (green), monocytes (red), granulocytes (blue) and a novel $MIL4^{-}SSC^{large}$ population (orange). As shown in Fig. 2D, we further separated the $MIL4^{-}SSC^{large}$ subpopulation (orange) by gating with MIL4/CT7 into $MIL4^{-}CT7^{high}$ cells (approximately 84±6%) of the $MIL4^{-}SSC^{large}$ cells and $MIL4^{-}CT7^{-}$ cells (approximately 16±3%, gated population of Fig. 2D). Thus, we separated guinea pig PBL into 5 distinct subpopulations.

3.3. Morphological confirmation of the five leukocyte fractions isolated by cell sorting

We first sorted the green, red and blue leukocyte fractions in Fig. 2A with a FACS Vantage and morphologically confirmed them as guinea pig lymphocyte, monocyte and granulocyte fractions, respectively, using a conventional May–Giemsa staining procedure (data not shown). Next, we further sorted the two leukocyte fractions in Fig. 2D with the cell sorter and used May–Giemsa staining to reveal that the $MIL4^{-}SSC^{large}CT7^{-}$ population, the gated subpopulation in Fig. 2D, was comprised of eosinophils and basophils (Fig. 3A). However, another $MIL4^{-}SSC^{large}CT7^{high}$ population, unique to guinea pigs, generally contained an inclusion body in the cytoplasm

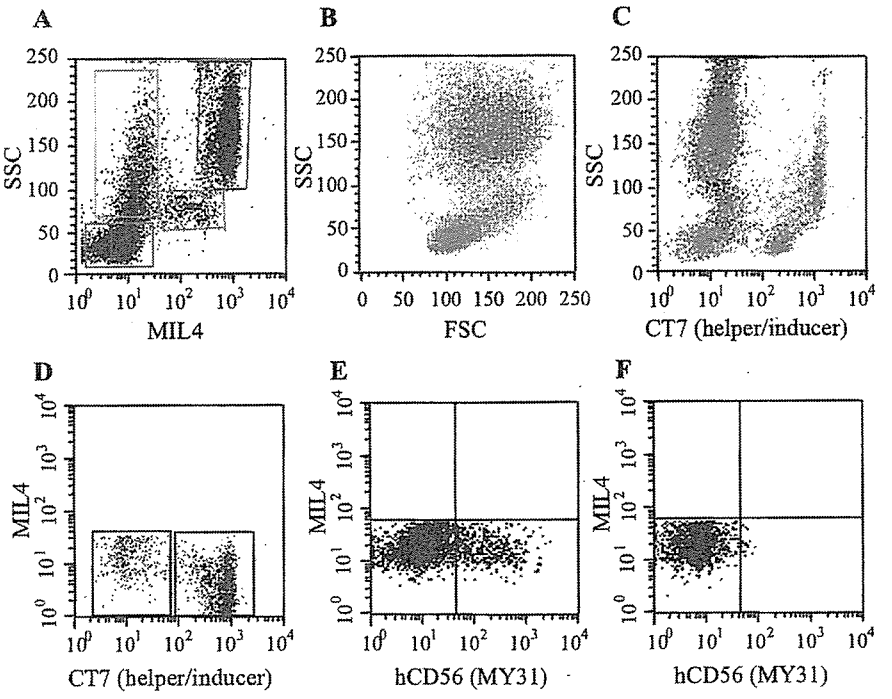

Fig. 2. Consecutive flow cytometry with MIL4/SSC and MIL4/CT7. Guinea pig leukocyte fractions were separated using consecutive flow cytometry with MIL4/SSC and MIL4/CT7. First, dead cells were excluded using propidium iodide and viable cells were gated using MIL4/SSC. Each of the gated colors in A is equivalent to the same color dots indicated by FSC/SSC (Fig. 2B) and CT7/SSC (Fig. 2C) and have been retrospectively confirmed by morphological analysis: lymphocytes (green), monocytes (red), granulocytes (blue) and MIL4$^-$SSC^{large} cells (orange). Separation of guinea pig PBL by MIL4/SSC 2-parameter flow cytometry (A), FSC/SSC (B), and CT7 (helper inducer)/SSC 2-parameter flow cytometry (C). Panel (D) shows further separation of the gated MIL4$^-$SSC^{large} population in panel A with MIL4/CT7 2-parameter flow cytometry into a CT7$^+$ fraction (right-gated fraction with inclusion body-positive cells) and a CT7$^-$ fraction (left-gated fraction with eosinophils plus basophils). (E) Cross-reactivity of human CD56 antibody with a CT7$^+$ cell fraction containing inclusion bodies (as described in Fig. 3B) was analyzed by gating with MIL4/hCD56. Approximately 6% of them were positive for hCD56. (F) In contrast, CT7$^-$ cells, consisting of eosinophils plus basophils, showed no cross-reactivity with MIL4/hCD56 cells. Only viable cells were analyzed, with dead cells excluded using propidium iodide.

(Fig. 3B). The MIL4$^-$SSC^{large}CT7^{high} cells had a mean diameter of approximately 12 μm, with nearly half of the cells containing a large cytoplasmic inclusion body. The inclusion body had an internal structure characterized by agglutinated or dispersed pachychromatic and reddish microparticles.

Thus, nearly 50% of the MIL4$^-$SSC^{large}CT7^{high} leukocytes were morphologically indistinguishable from previously reported Kurloff cells (Debout et al., 1984). The remaining half of the cell population had a smaller inclusion body with a mean diameter from 2 to 9 μm and an internal structure characterized by agglutinated, pachychromatic or reddish microparticles. Thus, this morphological characterization clearly confirmed five leukocyte fractions for the guinea pig: the lymphocyte, monocyte, neutrophil, the eosinophil plus basophil fraction in addition to the inclusion body-positive cell fraction that seemed unique to guinea pigs.

The unique MIL4$^-$SSC^{large}CT7^{high} leukocytes in the non-gated population of Fig. 2D were found to be identical with the gated SSC^{large}CT7^{high} population in Fig. 1A-b, as expected (data not shown). The distribution of the unique cells was also traced by two- parameter analysis with FSC/SSC. We found the cells distributed throughout the neutrophil (11.09 ±9.42% of neutrophils of the blue cell fraction in Fig. 2B), monocyte (30.45±11.22% of monocytes of the red cell fraction in Fig. 2B) and lymphocyte (1.27 ±0.75% of lymphocytes of the green cell fraction in Fig. 2B) cell populations.

Thus, our findings confirm previous observations that the PBL fractions of guinea pig leukocytes were very difficult to clearly identify using the traditional FSC/SSC flow cytometric assay. However, when guinea pig PBL were analyzed using a consecutive flow cytometric assay using MIL4/SSC followed by MIL4/CT7, we were able to accurately identify five

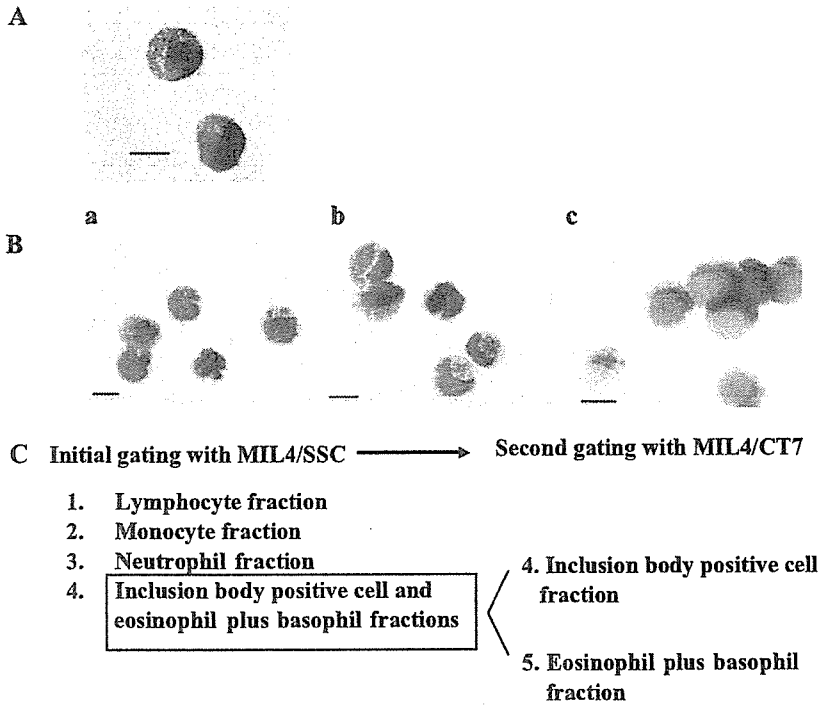

Fig. 3. Morphological character of MIL4$^-$SSC^{large}CT7^{negative} cells and MIL4$^-$SSC^{large}CT7^{high} cells. The MIL4$^-$SSC^{large}CT7^{negative} and MIL4$^-$SSC^{large}CT7^{high} cells shown in Fig. 2D were purified using flow cytometric sorting from normal guinea pig PBL. (A) May–Giemsa staining of MIL4$^-$SSC^{large}CT7^{negative} cells revealed eosinophils (upper cell in panel A) and basophils (lower cell in panel A). (B) MIL4$^-$SSC^{large}CT7^{high} cells stained by May–Giemsa (B-a and B-b) and PAS (B-c). Bar sizes in the panels are diameters of 10.0 μm. (C) Summary of fractionation procedure of guinea pig leukocytes. The guinea pig leukocytes were separated into five fractions by flow cytometric analysis. Using MIL4/SSC, we separated guinea pig leukocytes into five fractions: (1) lymphocytes, (2) monocytes, (3) neutrophils, (4) inclusion body-positive cells, and (5) eosinophils plus basophils. The MIL4$^-$SSC^{large} fraction was further separated into CT7$^+$ (inclusion body-positive cell), CT7$^-$ (eosinophil plus basophil) populations by MIL4/CT7 2-parameter flow cytometry.

subtypes of leukocytes including the novel MIL4$^-$SSC^{large}CT7^{high} cells (Fig. 3C).

3.4. The low cross-reactivity of human natural killer cell marker CD56 with MIL4$^-$SSC^{large}CT7^{high} leukocytes

Since nearly 50% of the unique MIL4$^-$SSC^{large}CT7^{high} leukocytes seemed to be morphologically indistinguishable from Kurloff cells in that they contained large cytoplasmic inclusion bodies in the cytoplasm, we investigated whether they would react with antibodies against human natural killer cells CD56 (Pouliot et al., 1996). Of the various human CD56 clones [leu19 (MY31, NCAM16.2 BD), human CD56 (B159 Pharmingen), human CD56 (NKI-nbi-1 Nichirei) and human CD56 (NKH-1 Coulter)] we used to screen for cross-reactivity with the SSC^{high}MIL4$^-$CT7^{high} guinea pig cells (right-gated subpopulation of Fig. 2D), only leu19 (MY31, BD) proved to be cross-reactive, reacting with 6.3±2.4% of the unique leukocyte population (Fig. 2E). In contrast, MIL4$^-$SSC^{large}CT7$^-$ leukocytes (left gated subpopulation of Fig. 2D) did not react with any of the human CD56 clones (Fig. 2F). Thus, only a very small subpopulation of the SSC^{high}MIL4$^-$CT7^{high} guinea pig cells cross-reacted with a human CD56 NK cell marker, a far lower rather of cross-reactivity with human CD56 antibodies than seen with Kurloff cells (Pouliot et al., 1996).

3.5. Cell surface analysis of the unique leukocyte fraction of guinea pigs

To characterize the unique cell fraction MIL4$^-$SSC^{large}CT7^{high}, leukocytes were further characterized by flow cytometry and were found to be reactive with helper/inducer antigens (CT7, H155), PanT (CT5, H159), CD45 (IH-1, H201), Thy-1 (H154), anti-asialo GM1 and FcR (20ED7) antibodies, but not with CD8 (CT6), B cell (Msgp9), IgM (31D2), MHC class II (R27E7) and MIL4 antibodies (Fig. 4). Moreover, these leukocytes proved to be more reactive with

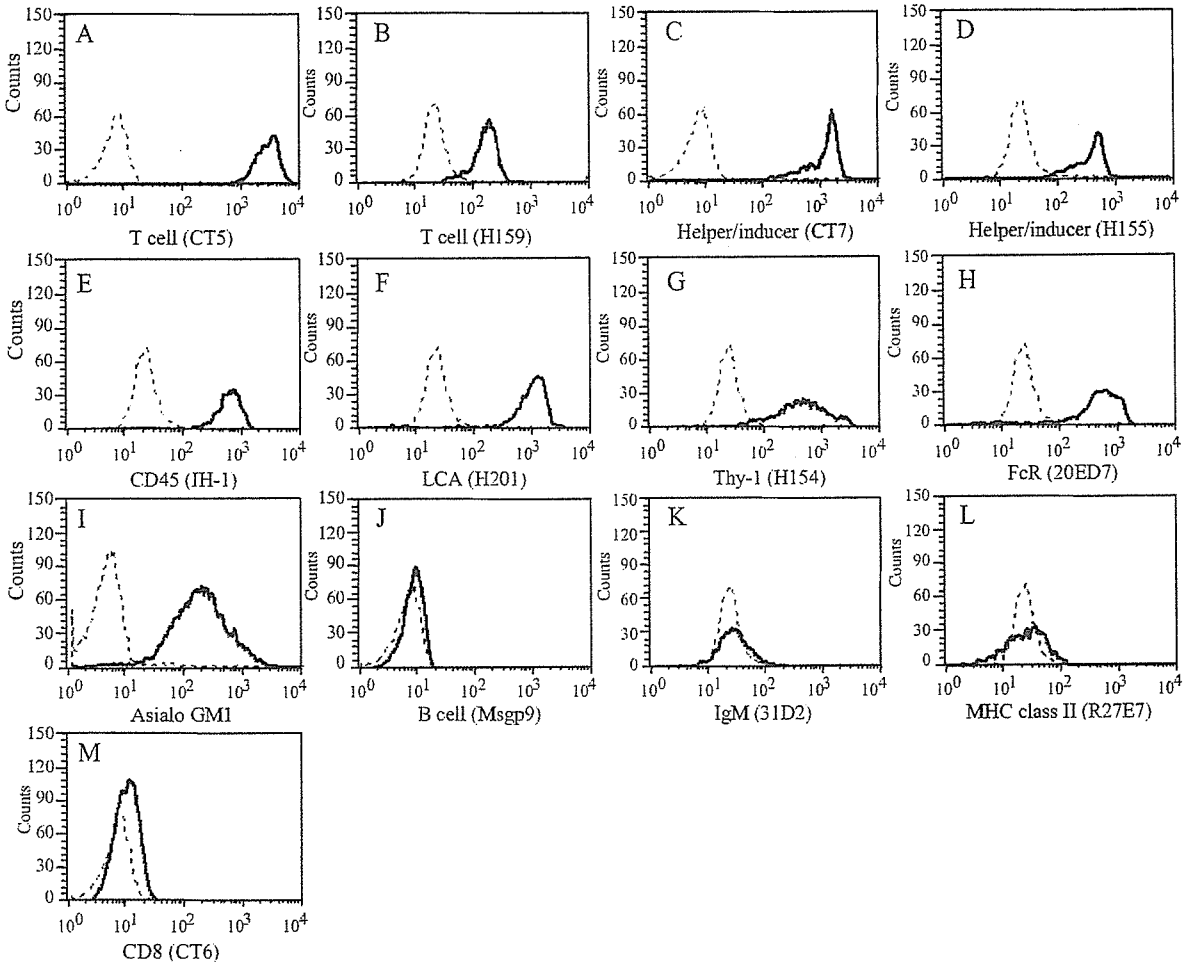

Fig. 4. Characteristics of the unique inclusion body-positive cells of guinea pig PBL as determined by flow cytometry. The MIL4^{-}SSC^{large}CT7^{high} fraction of PBL (orange fraction in Fig. 2) was further evaluated using the respective antibodies for the antigens expressed on their cell surface. Panels A, C, J, and M represent flow cytometric profiles of MIL4^{-}SSC^{large}CT7^{high} cells gated by APC-MIL4 and PE-CT7 followed by FITC-specific antibodies. Moreover, panels B, D, E, F, G, H, I, K and L represent the profiles of the unique cells gated by APC-MIL4 and FITC-CT7 followed by PE-specific antibodies.

helper/inducer antigens (CT7 or H155 mAbs) than lymphocytes in either peripheral blood (Fig. 2C) or spleen (data not shown). Guinea pig monocytes/macrophages were negative for reactivity with the CT7 antigen. Thus, because histograms of the various cell surface markers on the MIL4^{-}SSC^{large}CT7^{high} cells showed a haploid phase, we surmised that the unique cell population was a single leukocyte fraction of guinea pigs.

3.6. Normalization of the five leukocyte fractions in lymphoid tissues

The five leukocyte fractions of guinea pigs were distributed to the various lymphoid tissues by gating cells with MIL4/SSC followed by MIL4/CT7 (Fig. 5 and Table 2). As shown in Fig. 5A and Table 2, leukocyte fractions of spleen were gated as well as those of PBL. MIL4^{-}SSC^{large}CT7^{high} leukocytes were also similarly sorted to have a unique inclusion body in the cytoplasm (Fig. 5A-c). Lymph nodes (LN) and thymus were predominantly populated by lymphocytes and contained only 0.4% and 0.2% of MIL4^{-}SSC^{large}CT7^{high} leukocytes, respectively. BM cells were mainly comprised of myeloblasts and erythroblasts which were difficult to gate. In contrast, the MIL4^{-}SSC^{large}CT7^{high} leukocytes were clearly separated and they contained 1.6% of the total cells. Liver cells were also difficult to gate, but the unique cells in the liver could be separated using this consecutive flow cytometric procedure and were found in 4.3% of liver cells. The frequencies of these cells were 9.9% and 16.6% in peripheral blood and

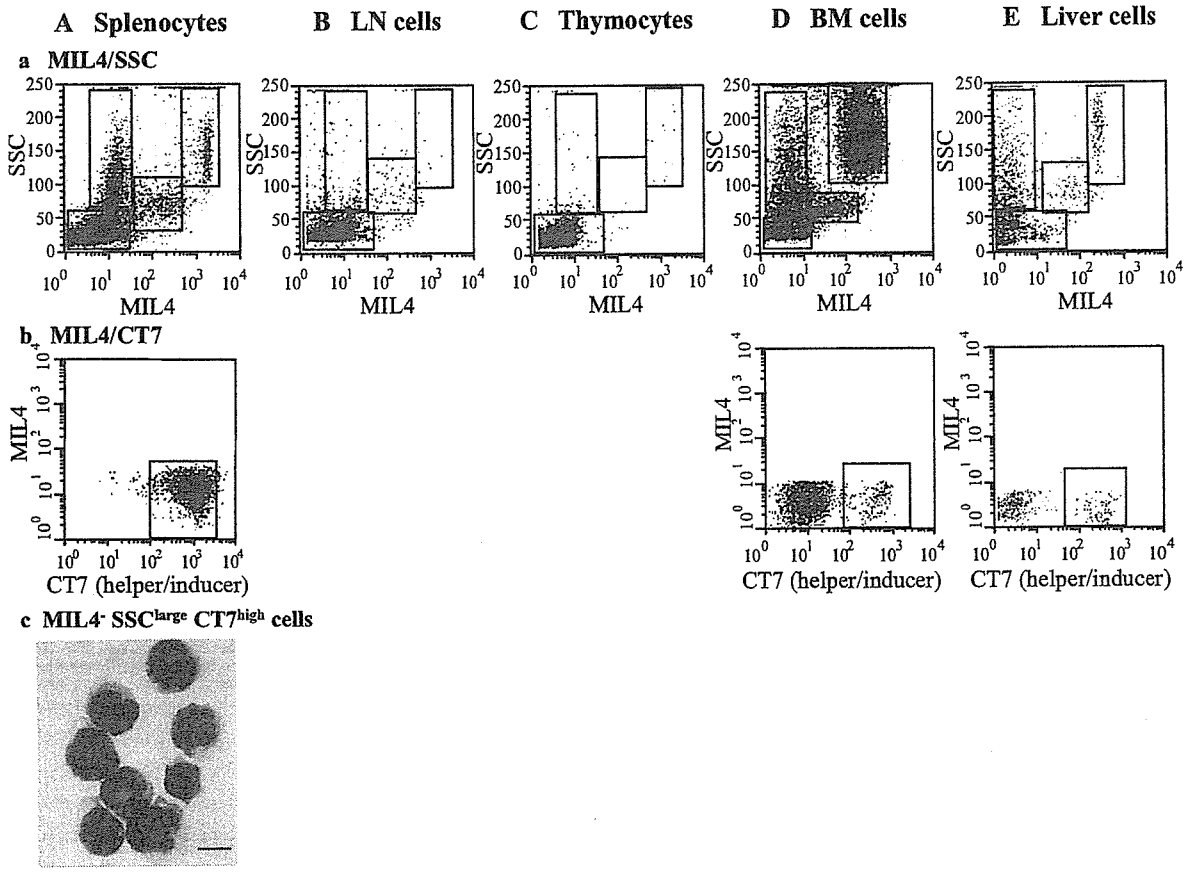

Fig. 5. Distribution pattern of leukocyte fractions in various guinea pig tissues. Cells isolated form various tissues of normal guinea pigs were analyzed by the novel two-parameter cytometric procedure: the MIL4/SSC parameter (a), followed by the MIL4/CT7 parameter (b). $MIL4^{-}SSC^{large}CT7^{high}$ cells (c) from spleen were sorted and their morphological character confirmed using May–Giemsa staining. Tissue distribution of guinea pig leukocyte fractions are shown for spleen (A), lymph node (B), thymus (C), bone marrow (D) and liver (E). Dead cells were excluded using propidium iodide and viable cells were analyzed.

spleen, respectively (Table 2 and Fig. 5A). Thus, the $MIL4^{-}SSC^{large}CT7^{high}$ leukocytes are abundant in the spleen and PBL, but are comparatively rare in the LN, BM and thymus of healthy guinea pigs. Fewer eosinophils and basophils are found in spleen cells (non-gated population in Fig. 5A-b) than in PBL (gated population in Fig. 2D). In summary, in our study we have been able to identify and separate the five fractions of guinea pig leukocytes, not only in PBL but also in the other lymphoid tissues. The novel flow cytometric technique presented here promises to dramatically improve the immunological analysis of guinea pig animal models.

4. Discussion

In this study, we found that MIL4, which is an antibody against porcine peripheral blood neutrophils, eosinophils and basophils (Haverson et al., 1994), cross-reacted with guinea pig neutrophils and moderately reacted with guinea pig monocytes/macrophages, but did not recognize lymphocytes, eosinophils, basophils and the $MIL4^{-}SSC^{large}CT7^{high}$ cells. Based on this observation, we distinguished guinea pig leukocyte fractions using consecutive flow cytometry of MIL4/SSC followed by MIL4/CT7 gating. The CT7 mAbs used for leukocyte typing in this study were screened and selected based on reports that CT7 recognizes a surface helper/inducer T cell (Steerenberg et al., 1991; Tan et al., 1985).

Light-scatter gating, commonly used to differentiate leukocyte cell types in humans and other species, proved ineffective for guinea pig cell differentiation because the $MIL4^{-}SSC^{large}CT7^{high}$ cells were widely dispersed throughout the light-scatter profiles of guinea pig granulocytes, lymphocytes and monocytes/macrophages. Using a traditional FSC/SSC gate, with the $MIL4^{-}SSC^{large}CT7^{high}$ cells located between the

Table 2
Mean percentage of leukocyte fractions in various tissues of normal guinea pigs obtained by using MIL4/SSC and MIL4/CT7 parameters[a]

Tissue	Leukocyte fraction	Mean	±SD
Blood	Lymphocytes	48.7	14.1
	Monocytes	4.7	2.6
	Neutrophils	33.6	14.2
	Inclusion body-positive cells	9.9	6.4
	Eosinophils and basophils	1.8	0.6
Spleen	Lymphocytes	68.2	7.2
	Monocytes	4.4	1.6
	Neutrophils	3.1	1.4
	Inclusion body-positive cells	16.6	6.2
	Eosinophils and basophils	0.7	0.4
Thymus	Lymphocytes	96.5	0.6
	Monocytes	0.6	0.2
	Neutrophils	0.1	0.0
	Inclusion body-positive cells	0.2	0.0
	Eosinophils and basophils	0.0	0.0
LN	Lymphocytes	94.8	1.4
	Monocytes	1.1	0.4
	Neutrophils	0.1	0.1
	Inclusion body-positive cells	0.4	0.0
	Eosinophils and basophils	0.0	0.0

[a] Leukocytes of guinea pigs were gated into 5 fractions by flow cytometry using MIL4/SSC followed by MIL4/CT7. The MIL4$^-$SSC^{large} fraction was separated into CT7$^+$ and CT7$^-$ cells. Data are expressed as the mean±SD.

monocyte and granulocyte populations on the SSC, clean leukocyte fractions could not be obtained, as MIL4$^-$SSC^{large}CT7^{high} cells contaminated approximately 30% of the monocyte and 10% of the granulocyte fractions, as well as a minor fraction of lymphocytes. Since non-lymphocytes are usually gated based on light scatter, and lymphocytes are gated based on CD45 fluorescence (Nicholson et al., 1996), we tried to gate guinea pig lymphocytes using CD45/SSC. This alternative gating approach also failed to effectively separate guinea pig leukocyte fractions, this time because each fraction's cells were stained with the same intensity of CD45 (data not shown).

The MIL4$^-$SSC^{large}CT7^{high} population contained inclusion bodies of varying sizes, with nearly half having the large inclusion body. This finding suggests that the MIL4$^-$SSC^{large}CT7^{high} cells containing the large inclusion bodies are similar to KC cells containing a large PAS-positive inclusion body. KC cells are also reported to be circulating mononuclear cells with NK-like function in vitro (Berendsen and Telford, 1966: Debout et al., 1984, 1999). The surface phenotype of MIL4$^-$SSC^{large}CT7^{high} was highly positive for helper/inducer antigens (CT7 or H155), T cell antigens (CT5, H159), Thy-1 (H154), CD45 (IH-1, H201), asialoGM1 and FcR (20ED7). However, it was negative for CD8 (CT6), B cell antigens (Msgp9), IgM (31D2), MHC Class II (R27E7) and MIL4. Thus, the surface of MIL4$^-$SSC^{large}CT7^{high} cells exhibited some NK and some T cell markers, but exhibited neither B cell markers nor definite CD3 antigen.

Previously, KC cells, noted for their large cytoplasmic inclusion bodies, were purified from the spleen of estradiol-inoculated guinea pigs by Percoll-gradient centrifugation (Debout et al., 1991; Pouliot et al., 1996). Most of the cells were shown to be cross-reactive with human NK cell marker CD56, suggesting that the cells might be homologues of human NK cells (Pouliot et al., 1996). Furthermore, KC cells were reported to be positive for CT7, but negative for 8EB7, CT6 and B-lymphocyte lineage antigens (CT10, IgM) (Debout et al., 1991). In contrast, only clone MY31 (B.D.) of the CD56 antigen reacted with the MIL4$^-$SSC^{large}CT7^{high} population of spleen cells from normal guinea pigs, and that only at a rate of 6%, as described above. This low cross-reactivity with human CD56 for the MIL4$^-$SSC^{large}CT7^{high} cells compared to that for purified KC cells is not surprising, since KC cells make up only a part of one subpopulation of MIL4$^-$SSC^{large}CT7^{high} cells, that with large inclusion bodies. We have here introduced methods for identifying and isolating the MIL4$^-$SSC^{large}CT7^{high} cells contained in approximately 10% of PBL and 17% spleen cells.

Although FSC/SSC gating puts both the eosinophil and basophil as well as the monocyte subsets in the same fraction (data not shown), we made it possible to separate the two guinea pig leukocyte fractions. An identification of eosinophils and basophils of guinea pigs will be beneficial to the study of immediate-type hypersensitivity reactions in guinea pig models. Therefore, the identification of five distinct subtypes of guinea pig leukocytes, including one unique MIL4$^-$SSC^{large}CT7^{high} cell type, paves the way for examining the role of each of these cell types in the infections and inflammatory conditions that can be uniquely studied in guinea pigs.

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