Cellular and Soluble CD4 Measurements in Cynomolgus Monkeys

Gareth Owain EVANS and Rajni FAGG

Drug Safety Evaluation, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, UK.

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Anti-human CD4 monoclonal antibodies have been successfully used to label T-lymphocytes in Cynomolgus monkeys by two different methods. A magnetisable bead separation was used prior to immunogold labelling of lymphocytes in one of the methods.

In addition, an assay for soluble CD4 has been applied to the sera of these monkeys. —
KEY WORDS : CD4, Cynomolgus monkey, immunogold labelling, soluble CD4, T-lymphocytes

There is evidence that lymphocytes from non-human primate species cross react with monoclonal antibodies (MABs) which show specificity towards human lymphocytes, and this includes MABs for the cluster differentiation 4 (CD4) antigen [1–11]. This CD4 antigen is expressed on the cell membranes of thymus derived lymphocytes, helper/inducer T cells and to a lesser extent on monocytes and macrophages in man [12], and it is used as a marker for disease progression and treatment in acquired immune disease syndrome (AIDS).

The objectives of this study were to measure CD4 reactive lymphocytes and the levels of soluble CD4 in peripheral blood from cynomolgus monkeys (*Macaca fascicularis*) using different techniques. The lymphocytes were enumerated using two different cell counting methods: an immunogold labelling technique with microscopic counting method of labelled cells and a flow cytometric method with fluorescein isothiocyanate (FITC - conjugated MAb). A 'sandwich' enzyme linked immunoassay for soluble CD4 (sCD4) designed for use with human sera was applied to sera from cynomolgus monkeys.

Materials and Methods

Animals and blood samples: Blood samples were collected from the femoral veins of inbred cynomolgus monkeys, following sedation with Ketamine: the male and female animals were aged 2 to 6 years. The blood was added to

tubes containing dipotassium ethylenediamine tetraacetic acid (1.5 to 2mg/ml of blood) or into plain tubes for serum separation.

For sCD4 assays, eighteen sera were obtained following centrifugation at 12,000g for 2 min. These samples were stored at -20°C prior to analysis. A separate study had shown that the samples appeared to give similar results when stored at -20°C for up to 28 days. Total white blood cell and lymphocyte counts: The total leucocyte count was determined using a Sysmex E5000 blood cell counter (Toa Medical Electronics Co. Ltd., Kobe, Japan). Total lymphocyte counts were carried out by microscopic examination, after staining blood films with a modified Polychrome Methylene blue reagent (Ames Laboratories, Stoke Poges, U. K.).

Immunogold labelling method: Immunogold labelling methods previously described, and used in this laboratory for labelling rat and canine T-lymphocytes [13–15] were modified by the introduction of Dynabeads™ for the separation of lymphocytes from the whole blood sample. The Dynabeads (product M450-CD4; Dynal AS, Oslo, Norway) are magnetisable polystyrene beads coated with a mouse anti-human CD4 MAb (anti-CD4 beads). These beads were used according to the manufacturer's instructions, and enabled the separation of cells which bind to the anti-CD4 beads; the detachment of the cells from the beads occurs during an overnight suspension period of 20 hr.

The cells were then labelled with a mouse

Table 1. T-lymphocyte, lymphocyte and leucocyte counts in cynomolgus peripheral blood samples

T-lymphocytes % Mean (SD) Range	Lymphocytes $\times 10^9/l$ Mean (SD) Range	Leucocytes $\times 10^9/L$ Mean (SD) Range
Group 1 (n=12)		
Immunogold method		
24 (3)	2.18 (1.13)	4.06 (0.92)
14 to 38	1.9 to 3.5	2.8 to 5.5
Group 2 (n=10)		
Immunogold method		
18 (4)	1.78 (0.43)	2.60 (0.58)
13 to 23	1.4 to 2.2	2.0 to 3.2
FITC method		
24 (6)		
17 to 33		

anti-human CD4 MAb (product MCA 481; Serotec Oxford, U. K.) and then with a goat anti-mouse IgG conjugated Immu-Mark™ gold probe (probe size 20nm, product 678552; ICN Biomedicals, High Wycombe, U. K.). Positively labelled cells with blue black gold particles were then counted using a light microscope, and the results were expressed as percentages of the total number of lymphocytes. FITC-flow cytometry method: FITC conjugated antibody mouse anti-human CD4 ($20\mu\ell$, product MCA 635F; Serotec, Oxford, U. K.) was incubated with whole blood ($100\mu\ell$) for 15 min at room temperature. Erythrocytes were removed by the addition of a lysing agent (FACS™ lysing solution, product 92-002; Becton-Dickinson, California, U. S. A.); red cell stroma was then discarded by centrifugation and washing with phosphate buffered saline. The FITC-labelled cells were then fixed using 1% paraformaldehyde prior to analysis by flow cytometry. A FACS™ IV cytometer (Becton-Dickinson, California, U. S. A.) was appropriately gated for lymphocyte counting. Negative controls were prepared using an antibody of the same isotype as the test antibody (mouse anti-human negative control, product MCA 690F; Serotec). The FITC labelled mouse anti human CD4 was from the same clone as used for immunogold labelling. Soluble CD4: The measurement was performed with Cellfree™ CD4 test kit (T Cell Sciences Inc., Cambridge, MA, U. S. A) according to the manufacturer's instructions. An anti-CD4 MAb is adsorbed onto microtitre plate wells

and samples and standards are added to the appropriate wells. Following washing procedure to remove non-reactive materials, horse raddish peroxidase conjugated anti CD4-MAb (HRPMAb) was added to form the 'sandwich'. Unbound HRPMAb was removed by washing and o-phenylene diamine was added to measure degree of bound peroxidase activity colorimetrically at 492nm. Six standards, containing a recombinant human CD4, were used to construct a calibration curve and determine the amount of sCD4 in the samples [16].

Using a pooled cynomolgus serum, the within-run coefficient of variation was 1.0% and the intra-run coefficient of variation was 0.83% at a level of $20 \times 10^3 U/l$ (n=24). Recovery experiments were performed by assaying three cynomolgus sera before and after the addition of the $40 \times 10^3 U/l$ human recombinant standard (supplied with the kit) to these samples. The original mean values and percentage recoveries were 15, 17 and $21 \times 10^3 U/l$ and 90, 80 and 68% respectively, after correction for dilution of the monkey plasma with the recombinant standard.

Results

Positively labelled CD4 lymphocytes were observed in two groups of peripheral blood sample obtained from cynomolgus monkeys using the immunogold and immunofluorescence methods: the data obtained by both methods are shown in Table 1. Samples from Group 2 were analysed by both methods; the values

obtained by the flow cytometric method were slightly higher than those with the immunogold method. However these results were similar to those obtained in the first group with immunogold labelling.

The sCD4 values obtained for cynomolgus sera ranged from 6 to 27×10^3 U/l, with mean and standard deviation values being 21 and 7×10^3 U/l, respectively.

Discussion

Although mouse anti-human CD4 monoclonals do not always recognise CD4 epitopes in non-human primates [10,17], this study has demonstrated anti-CD4 reactivity in cynomolgus monkeys using two cell labelling techniques. One published report stated that there were no positively reactive cells with anti-human CD4/Leu3a [5], and another report suggested 33% of the lymphocytes were reactive with anti CD4/T4 MABs [2] in this species.

The introduction of a magnetic separation device for lymphocyte preparation appears to be a useful but time-consuming procedure. Of the two techniques, the FITC-flow cytometric method is preferred, but many laboratories still do not have access to such equipment. One advantage of the flow cytometric method may be the use of paraformaldehyde to fix cells particularly from immunocompromised animals with obvious or latent viral infections, although several other problems may occur with T-cell subset enumeration using flow cytometry [18,19]. There are insufficient data in this study to comment on age-related changes reported for other non-human primates [7,8], and we have not studied intra-animal physiological variations.

From these preliminary studies, it appears that sCD4 assays may be worthy of further investigation in disease states where levels of sCD4 may alter in cynomolgus monkeys. The range of values observed for cynomolgus monkeys was slightly narrower than that obtained for human sera (8 to 65×10^3 U/l) using the same assay.

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カニクイザルにおける細胞性および可溶性 CD4 の測定

エバンス, G. O. ・ ファッグ, R.

ウエルカム研究所薬剤安全性評価部門

抗ヒト CD4 モノクローナル抗体を使用して、2 つの異なる方法により、カニクイザルの T リンパ球を標識することができた。そのうちの一方では、磁気化できるビーズを用いた分離法を行った後、リンパ球をイム

ノゴールド（金結合抗体）によって標識した。さらに、可溶性 CD4 の定量をこれらのサル血清について行った。