3. MATERIALS AND METHODS

3.1. Preparation of anti-bovine lymphocyte serum.

The anti-bovine lymphocyte serum (ABLS) used in these experiments was produced by Dr J.G. Kreeftenberg and F. Leerling from the National Institute of Public Health, Bindhoven, the Netherlands and was raised in four horses according to the method described by Monaco et al. (1966) with some modifications. Lymphocytes were derived from a thymus gland of a calf. The thymus gland obtained from a local slaughter house was collected in cold Hanks' BSS medium. On arrival in the laboratory the tissues were cleaned, defatted and homogenized in Hanks' BSS medium with a loose fitting Potter tube. The cell suspension was filtered through a double layer of sterile gauze to removed coarse thymus particles, washed twice and then resuspended in Hanks' BSS medium. The concentration was adjusted to 2.5 x 10^8 lymphocytes per ml. When not directly used the cell suspension was stored in liquid nitrogen. The cells were frozen in 10% horse serum and 10% dimethyl sulphoxide.

Adult clinically normal horses were used for immunization. Figure 1 describes the method of immunization. Each horse was first injected subcutaneously with 3.0 x 10^9 thymic cells in complete Freund's adjuvant. The booster immunization was given 3 weeks thereafter in two divided doses each with 1.5 x 10^9 cells administered intravenously on two successive
Fig. 1. The modified Monaco’s method for raising ABLS in a horse. The horse received subcutaneously $3 \times 10^9$ thymocytes in CFA on day 0, followed by two intravenous injections of $1.5 \times 10^9$ thymocytes in Hanks' BSS medium on day 23 and 24. The times of plasmaphereses are given in the figure.
days. Between day 10 and 24 after the last injection the horse was subjected to a series of series of plasmaphereses. After collection the serum was stored in \(-20^\circ C\) until used. The serum was used without inactivation or absorption with bovine erythrocytes.

Determination of cytotoxic and haemolysin titers.

The cytotoxic titer of the ABLS was determined by trypan blue dye exclusion test in the presence of rabbit complement in a microtiter plate. Fifty \(\mu l\) of two fold ABLS dilutions were incubated with 50 \(\mu l\) of bovine peripheral blood lymphocyte suspension in Hanks' BSS medium containing \(6.0 \times 10^6\) cells per ml and 50 \(\mu l\) of fresh rabbit serum (complement) diluted 1:10 in saline. After one hour incubation at \(37^\circ C\) the percentage of dead cells was estimated by the addition of one drop of 0.1% trypan blue. The titer was the reciprocal of the highest dilution which killed at least 50% of the bovine lymphocytes.

The haemolytic titer was also determined at two fold serum dilutions in glass tubes. The dilutions were incubated for one hour at \(37^\circ C\) with equal volumes of a 0.5% bovine erythrocyte suspension in saline and of a 1:20 dilution of guinea pig complement in saline. The titer was the reciprocal of the highest serum dilution that caused haemolysis.
The experimental calves.

Forty, four to six months old clinically normal and unrelated calves of the Dutch Friesian breed were used throughout these experiments. The calves were divided into four groups as described in table 1 and housed in isolated barns at the Central Veterinary Institute in Rotterdam, the Netherlands.
Table 1. Grouping of the experimental calves according to use.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of calves</th>
<th>Purpose</th>
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</table>
| I     | 17               | 1. determination of the dose of ABLS.  
2. the study of clinical, post mortem including histologic alterations caused by ABLS. |
|       | 10               | 1. the study of the haematologic response to ABLS.  
2. the study of the effect of ABLS on the humoral immune response following parenteral application of sheep red blood cells (SRBC)  
3. the observation of delayed hypersensitivity reaction following tuberculinization. The ABLS treated calves were sensitized by intravenous injection of Mycobacterium microti. |
|       | 10               | 1. the study of the haematologic response to ABLS.  
2. the observation of the effect of ABLS on the cell mediated immunity determined by skin grafting.  
3. the study of the effect of ABLS on the humoral immune response following tetanus toxoid injection. |
|       | 3                | 1. the study of the influence of ABLS on the ability of the lymphocytes to form blast cells following stimulation.  
2. the estimation of the effect of ABLS on the B and T cells. |
3.1. Group I

3.1.1. Determination of the doses of ABLS.

For this purpose 17 calves were used. These calves were divided into 5 subgroups as shown in table 2. The ABLS was injected subcutaneously at various quantities. Subgroups 4 and 5 comprising three calves were given normal horse serum as control.

From this experiment it was found that 1 ml ABLS per kg body weight was sufficient to depress the peripheral blood lymphocyte population, whereas the erythrocyte, haemoglobin and thrombocyte counts remained normal and the animals showed only slight clinical signs.

Table 2. Number of experimental calves in group I, dose and route of application of anti-bovine lymphocyte serum for the estimation of the effective dose.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Number of calves</th>
<th>Dose/kg body weight</th>
<th>Number and mode of injections</th>
<th>Days of intervals</th>
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<tbody>
<tr>
<td>1.</td>
<td>4</td>
<td>7.50-10 ml ABLS</td>
<td>3 subcutaneous</td>
<td>4 and 2</td>
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<td>2.</td>
<td>3</td>
<td>3.00-6 ml ABLS</td>
<td>2</td>
<td>1</td>
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<tr>
<td>3.</td>
<td>7</td>
<td>0.75-1 ml ABLS</td>
<td>2</td>
<td>1</td>
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<tr>
<td>4.</td>
<td>1</td>
<td>5 ml normal horse serum</td>
<td>2</td>
<td>1</td>
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<tr>
<td>5.</td>
<td>2</td>
<td>3 ml</td>
<td>2</td>
<td>1</td>
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3.3.1.2. The clinical and post-mortem observations.

The calves were observed daily for clinical symptoms and temperatures were taken twice a day. The animals that succumbed were necropsied and a study of the gross and histologic alterations were made. For histological examinations pieces of organs including lymph node, thymus gland, lung, heart, liver, spleen, kidney, intestine, bone marrow and brain were collected and fixed in 10% buffered formalin. Thereafter the tissues were processed according to standard histological techniques. Sections of 5 microns were stained with routine haematoxylin and eosin. Other staining methods such as van Gieson and P.A.S. were applied when necessary.

3.3.2. Group II.

This group consisted of 7 ABLS treated calves and 3 controls. They received ten subcutaneous injections of ABLS at one day intervals at a dose of 1 ml per kg body weight. The injections were given at the left and right sides of the neck. The control calves were left untreated.

3.3.2.1. Haematological examinations.

Prior to the initiation of the experiments, haematological examinations were performed twice a week for two successive weeks for the estimation of the normal blood counts of the animals. After ABLS treatment the haematological
examinations were performed at 6 hours, 24 hours and then at one day intervals until seven times following the last injection.

Blood was obtained by jugular veni-puncture and collected in a tube containing a solution of sodium ethylene-diamine-tetra-acetic acid (EDTA) to prevent coagulation to a final concentration of 1 mg EDTA per ml blood.

For cellular counts and haemoglobin determinations 20 μl EDTA blood was diluted in 10 ml Isoton II solution (Coulter Electronics, Gmbh, 4150 Krefeld, Gajlingsfad 53, W. Germany) and 100 μl of this 1:500 diluted blood was suspended in 10 ml Isoton II solution (1:50,000) for erythrocyte counts and haematocrit determinations. For leucocyte counts 3 drops of Zapoglobine solution (Coulter Electronics Ltd, Coldharbour Lane, Harpenden, Herts, England) were added to the remaining suspension in order to lyse the erythrocytes.

The thrombocyte counts were determined by using the Coulter Platelet Kit (Coldharbour Lane, Harpenden, Hertz, England). Approximately 0.1 ml of EDTA blood was aspirated into a special plastic sedimentation tube and allowed to settle for 2 - 3 hours. The tube was then cut at the junction of the red cells and the plasma. The plasma rich thrombocytes was sucked into a 3,3 μl pipet and diluted in 10 ml Isoton II solution.

The leucocytes, erythrocytes and thrombocytes were counted in a Coulter Counter model ZF (Coulter Electronics...
Ltd, Coldharbour Lane, Harpenden, Herts, England), which was provided with a haemoglobin meter and haematocrit meter. The latter was connected with the Coulter Counter. When the erythrocytes were counted the haematocrit meter determined the haematocrit percentage at the same time.

The haemoglobin was determined with the haemoglobin meter using the same blood dilution as for the leucocyte count containing Zap-oglobin solution.

The leucocytes were differentiated in blood smears stained after May Grünwald and Giemsa (E. Merck, Darmstadt) for respectively 5 and 15 minutes. Differentiation was carried out with a Carl Zeiss light microscope using oil immersion at 600 x magnification.

3.3.2.2. The study of the effect of ABLS on the humoral immune response following parenteral application of sheep red blood cells (SRBC).

3.3.2.2.1. Preparation, dose and application of SRBC.

Normal sterile sheep blood in Alsevere (1:1) supplied by the Production Department of the Central Veterinary Institute, Rotterdam, was centrifuged at 340 g for 10 minutes and the packed cells were washed three times in sterile saline. After washing a final suspension containing 75.0 x 10^6 SRBC per ml was prepared in Hanks' BSS medium. Forty eight hours after the first administration of ABLS, each ABLS treated and control
The antibody titer against SRBC was determined by the direct haemagglutination test. Normal sheep blood in Alsever (1:1) was centrifuged at 340 g for 10 minutes and the pellet was washed 3 times in saline. After washing the haemoglobin titer was determined and adjusted to 16.0 mg % with saline and this concentration was considered as a 100% SRBC suspension. From this suspension a 1% SRBC suspension was prepared in a buffer consisting of 80% nutrient gelatin buffer (400 mg nutrient gelatin dissolved in 1 liter distilled water), 20% veronal buffer and 0.5% inactivated bovine foetus serum which was previously absorbed with SRBC. Two fold dilutions of the test sera were made in this buffer in microtiter plates. To 50 μl of the serum dilutions 50 μl of the 1% SRBC suspension was added and after sealing with a plastic tape deck the microtiter plates were mechanically shaken and left for 1 hour at room temperature. Thereafter the plates were further incubated for 1 hour in an incubator at 37°C. The titer of the antibody against SRBC was the reciprocal of the highest dilution that still showed agglutination.
3. Observation of delayed hypersensitivity reaction by tuberculination.

For testing the influence of ABLS on this type of delayed hypersensitivity reaction all ABLS treated and control calves were injected intravenously with 1 ml of a *Mycobacterium microti* suspension containing $3.0 \times 10^5$ viable cells units per ml at 24 hours after the administration of the first ABLS.

The classical hypersensitivity skin test was performed 4 weeks after the administration of *Mycobacterium microti*. This was done by injecting intradermally all ABLS treated and control calves with 0.1 ml of bovine and avian tuberculin. Each calf thus received 5000 TU bovine tuberculin PPD and 2000 TU avian tuberculin PPD on the right side of the neck. Before the tuberculin was administered the thickness of the skin was measured with a cutimeter. The skin reaction was determined 72 hours after the tuberculin injection by measuring the increase in the thickness of the skin at the site of injection.

3.3.3. Group III.

- The study of haematologic response to ABLS (see group II c.)
- Observation on the cell mediated immune response by skin grafting.
3.3-3.2.1. Treatment of the calves before skin grafting.

Animals of group III consisted of 10 calves were divided into 6 ABLS treated calves and 4 controls. The ABLS treated calves received ten injections of 1 ml ABLS per kg body weight at one day intervals. The control calves were injected with normal horse serum at the same dose and also at one day intervals four times. The administration of ABLS and normal horse serum was started two days before grafting (day -2) on the day of grafting (day 0) and every two days thereafter. Table 3 shows the donor-recipient pairs of calves in skin grafting.

Table 3. Donor-recipient pairs of calves in skin grafting.

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<td><strong>ABLS TREATED</strong></td>
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<td>123</td>
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<td>250</td>
<td>123</td>
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<tr>
<td><strong>Control</strong></td>
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3.3.2.2. Skin grafting

3.3.2.2.a. Preparation of the calves.

Twenty four hours before skin grafting food was withheld from all calves. The donor skin side and the grafting bed side were cleanly shaven and washed with water containing cytopogen (Gist-Brocades NV, Delft, the Netherlands) and disinfected with 70% ethanol. The calves were sedated by injection of 1 ml 2% Rompun solution (Bayer) intramuscularly. General anaesthesia was carried out with Numbutal solution (Abbott Lab) at a dose of 0.2 ml per kg body weight, administered in two divided doses. The first dose was injected before the removal of the graft and the second was given prior to the preparation of the graft bed and its fixation.

3.3.2.2.b. Preparation of the grafts.

White skin was chosen for the grafts to facilitate evaluation and comparison with the skin surrounding the graft beds. Grafts were removed from the lateral side of the hind leg below the stifle joint. The graft was the size of a microscope slide (2.5 x 6 cm$^2$), which was used as a template (figure 11). A full thickness skin graft of this size was removed by incision and blunt dissection. After removal, the graft was divided into two equal pieces (2.5 x 3 cm$^2$) which were placed with the hairy side down on a sterile gauze in
a petri dish containing a small volume of Hanks' BSS solution and antibiotics. The grafts were cleaned by removing the adhering fat and connective tissues with scissors or scraping with a scalpel.

The donor's wound was sutured with No. 1 silk suture and the surgical wound was smeared with a thin layer of Socatyl paste (Ciba Geigy, Bazel, Switzerland). The wound was covered with a sterile gauze and finally with plaster tape (Figs. 12-13).

3.3.2.2. Preparation of the graft beds.

The back on the left and right side of the lumbar region was chosen as the area of the graft beds in the recipient calves. The left side was used for the allograft and the right side was for the control autograft. The area for the graft bed was shaved, cleaned and disinfected as described. The recipient skin to be removed for the graft bed was cut somewhat smaller than the replacing graft to ensure a good fit and to compensate for the slight retraction of the skin around the graft bed and the tendency of the graft to contract after incision. The skin was dissected at the level of the superficial fascia and bleeding was carefully controlled with adrenaline solution (Fig. 14). When the bleeding had stopped the grafts were placed on the fascial surface and fixed by suturing with No. 0 silk, using 14 sutures for each graft.
3.3.2.d. Treatment of the grafts.

Following fixation the grafts were covered with Nebacetin powder (H. Lunback & Co A/S, Copenhagen), a piece of paraffin impregnated gauze, a piece of dry gauze and then with Hansopor plaster, and thereafter fixed with plaster tape. Finally the backs of the calves were covered with a white piece of cloth, which was held in place by two elastic bandages around the waist and chest (Figs. 17-18). The grafts were inspected every two days and at the same time the paraffin-impregnated and dry gauze were changed. To keep the grafts moist they were smeared with udder cream (ICI Pharmaceuticals Division). The sutures were removed on day 7 after the operation.

The parameters used for the evaluation of graft survival were the general macroscopic appearance, pliability, the occurrence of haemorrhages and desiccation, decrease of graft size and hair growth. No microscopic evaluation was performed.

3.3.3. The study of the effect of ABLS on the humoral immune response following tetanus toxoid injection.

3.3.3.1. Origin and dose of tetanus toxoid.

Tetanus toxoid containing 10 limit flocculation (LF) per ml was obtained from the National Institute of Public
Health in Bilthoven, the Netherlands. Each ABLS and control calves were injected intramuscularly with 3 ml tetanus toxoid suspension 48 hours after the first administration of ABLS.

3.2. Determination of the antibody titer against tetanus toxoid in blood serum.

The serum from experimental animals was collected twice a week. These sera were titrated by a haemagglutination test as described by Mai and Rosin (1968).

Sheep blood in Alsevere (1:1) was centrifuged at 340 g for 10 minutes and the packed cells were washed 3 times in saline. After washing, a 10% erythrocyte suspension in saline was prepared. An equal volume of a 3% neutral formalin solution was added and the suspension was incubated for 18 to 20 hours at 37°C. After incubation the treated SRBC were washed in distilled water until the supernatant was clear, at least three times.

After washing a 10% suspension of the formalinized SRBC in PBS pH 7.2 was prepared. This suspension can be stored up to 6 months when kept at 4°C. A mixture of an equal volume of 0.005% tannic acid solution (5 mg tannic acid dissolved in 100 ml PBS pH 7.2) and the 10% formalinized SRBC suspension was prepared and incubated for 20 minutes at 37°C. After incubation the suspension was washed once in PBS pH 7.2 and a 10% suspension of the formalinized tanned erythrocyte was
made in PBS pH 7.2. Sensitization of the formalinized tanned SRBC was performed by adding an equal volume of 100 LF per ml tetanus toxoid solution and incubating for 20 minutes at 37°C. After incubation the suspension was washed 3 times in a 0.5% dilution of inactivated normal rabbit serum in PBS pH 7.2 and a final suspension of 0.25% of the tetanus-sensitized SRBC was prepared in the same fluid.

The test serum was diluted two fold in disposable polystyrene microtiter plates (Nutacon). To each well that contained 50 μl of the diluted serum an equal volume of the tetanus toxoid sensitized formalinized tanned SRBC suspension was added. After covering with plastic tape decks the microtiter plates were mechanically shaken for 10 - 15 minutes and left at room temperature. The results were determined after 3 - 4 hours and the titer was the reciprocal of the highest serum dilution that still showed agglutination.

Group IV.

3.3.4.1. The study of the influence of ABLS on the ability of the lymphocytes to form blast cells following stimulation.

For this purpose three calves were used. Prior to the administration of ABLS, weekly lymphocyte stimulations were done for 3 weeks to obtain the normal counts per minutes (CPM) values of the calves (see further for details).
3.3.4.1.1. Demonstration of blastogenesis of lymphocytes.

The method of lymphocyte stimulation used was according to the technique reported by Muscoplat et al. (1974).

3.3.4.1.1.a. The collection of lymphocytes.

Twenty ml blood was collected by jugular venipuncture in a tube containing preservative-free heparin (E. Merck, Darmstadt) to a final concentration of 25 units heparin per ml blood. Subsequently the lymphocytes were isolated according to the method described by Boyum (1968). The tubes containing the heparinized blood was centrifuged at 340 g for 10 minutes. The supernatant plasma was decanted and the buffy coat was pipetted, placed in a tube and diluted with an equal volume of RPMI 1640 medium (Gibco, Lab). Subsequently 5 ml of the diluted buffy coat was layered on 5 ml Lymphoprep (density 1.077 g/ml, Nyegaard & Co A/S, Oslo, Norway) in 12 ml glass tubes with rubber stopper. The tubes containing the blood on Lymphoprep was centrifuged at 1100 g for 25 minutes at room temperature. The cells in the interface between the plasma and the Lymphoprep were collected and placed in 25 ml glass tubes with cap and were washed twice in RPMI 1640 medium and counted in Bürkit counting chambers. The cell viability was determined by the addition of 0.1% trypan blue dye solution. The cell population collected in this manner consisted of more than 95% lymphocytes and with
4.3.4.1.1.b. Culture medium and mitogen.

The culture medium was composed of ready made RPMI 1640 containing L-glutamine and Hapes (Gibcо Lab). To this medium 20% of inactivated foetal bovine serum (FBS, Gibcо Lab), 100 units of penicilline sulfate and 100 micrograms of streptomycin per ml was added.

The mitogens used were Phytohaemagglutinin (PHA, Wellcome) and Pokeweед mitogen (PWM, Gibcо Lab). These were dissolved in 5 ml distilled water as prescribed. The PHA solution was diluted 1:4 in culture medium and 10 μl of this dilution was used for each culture. The PWM solution was diluted 1:8 in culture medium and 10 μl was applied for each culture.

4.3.4.1.1.c. The cell cultures.

After counting, the cell suspension was adjusted to 1.4 x 10⁶ cells per ml. The cell cultures were prepared in cell culture microtiter plates (Nutacon) with round bottoms. Each well was filled with 10 μl diluted mitogen and 150 μl cell suspension so that each culture contained 2.1 x 10⁵ lymphocytes. Each sample was set up in triplicate with the same amount of controls containing no mitogen. After filling the wells the microtiter plates were covered with a plastic
tape cover and incubated at 37°C for 72 hours for the PHA stimulation and for 120 hours for the PWM. Eighteen hours before harvesting 50 µl of RPMI 1640 medium containing 1.0 uCi of methyl-3H-thymidins (3H-TdR, 6,7 Ci/moles) was added to each well. After 18 hours incubation the cultures were terminated by freezing until the cells were harvested for liquid scintillation counting. Harvesting of the cells occurred with a semiautomatic multiple sample harvester (Skatron, Lierbyen, Norway) on filter paper strips. After drying the filter paper strips were placed in 5 ml counting vials with 3 ml scintillation fluid. The vials were then placed in a scintillation counter Isocap 300 (Nuclear Chicago Division, Scarle) for counting.

The results were expressed as counts per minutes (CPM) or for comparison with the control as stimulation index (SI).

\[
SI = \frac{CPM\ of\ cultures\ with\ mitogen}{CPM\ of\ control\ culture}
\]

3.4.2. The estimation of the effect of ABLS on the B and T cells.

1. Isolation of lymphocytes.

Lymphocytes were collected from 50 ml heparinized blood, according to the technique already described. Contaminating erythrocytes were lysed by the addition of
0.83% NH₄Cl twice the amount of the lymphocyte suspension and left for 5 minutes at room temperature. Subsequently, the lymphocytes were washed twice in RPMI 1640 medium, resuspended in the same medium and counted. For T cells demonstration (by rosetting) the lymphocyte concentrations were adjusted to 2.0–3.0 x 10⁶ cells per ml in RPMI 1640 medium containing 20% FBS. For the demonstration of B cells the lymphocyte concentration was adjusted to 5.0–6.0 x 10⁶ cells per ml in RPMI 1640 medium without FBS.

3.3.4.2.1.a. Demonstration of T cells.

Thymus derived lymphocyte (T cell) determination was performed according to the method described by Wardley (1977) with minor modifications.

Sheep blood in Alsever (1:1) was washed 3 times in PBS pH 7.4 and a 4% suspension was prepared in 6% (w/v) dextran saline (Dextraven 150, Fisons Ltd, Loughborough, Leicestershire, England). Thereafter, 100 μl of this suspension was mixed with 150 μl lymphocytes suspension containing 2.0–3.0 x 10⁶ cells per ml in Nunk plastic tubes. This was followed by incubation for 30 minutes in a waterbath at 37°C. After centrifugation at 875 g for 5 minutes the cells were stored overnight (18 – 20 hours) at 4°C. The supernatant was discarded and the pellet was very carefully resuspended in 0.2 ml of 0.01% toluidine blue solution in PBS. Thereafter, the
suspension was dispersed in a Bürkit counting chamber and
counted in a light microscope. For each sample 200 lympho-
cytes were counted. Lymphocytes with three or more SRBC
adhering to the surface were considered as rosette forming
cells.

3.4.2. Demonstration of B lymphocytes.

Thymus independent lymphocytes or B cells with surface
immunoglobine (SIg) were determined by the method described
by Muscoplat et al. (1974) with some modifications. One
hundred μl of lymphocyte suspension containing 5.0–6.0×10^6
cells per ml was mixed with an equal volume of 1% parafor-
maldehyde solution (pH 7.2) for two minutes at room tempe-

ature. Hereafter, the cells were washed 3 times in PBS
pH 7.2. At each washing the cells were centrifuged at 875
g for 5 minutes.

Equal volumes of goat antibovine IgM and goat anti-
bovine IgG conjugates were mixed and diluted to 1:10 with
PBS pH 7.2. The antibovine IgG conjugate was obtained
commercially (Nordic, Tilburg, the Netherlands). Goat anti-
bovine IgM serum was donated by Drs Bokhout. It was conju-
gated with fluorescein-isothiocyanate (FITC) according to the
method described by Ressang et al. (1966).

After the last washing of the fixed lymphocytes the
supernatant fluid was discarded and the pellet was resuspended
in 0.1 ml conjugate mixture (1:10) and left at room temperature for 30 minutes. Thereafter, the cells were washed in PBS pH 7.2 as described. If not directly counted, the tubes with the cells were placed in an ice bath or at 4°C to prevent pinocytosis of the surface Ig-anti-Ig complexes.

A drop of the suspension was placed on a microscopic slide and covered with a coverslip. Cells with membrane fluorescence were counted with a Leica Diavert fluorescent microscope equipped with Plomeropak 2.2, a FITC 12 filter combination and a HBO 50 watt lamp as source, of blue incident light. Lymphocytes were cells showing membrane fluorescence. In each sample 200 cells were counted.