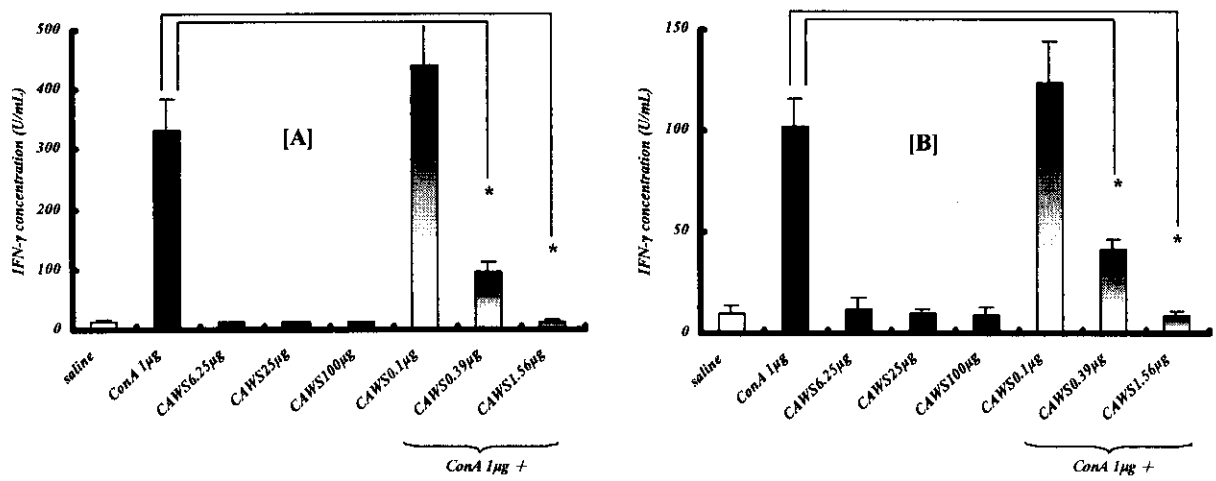


Fig. 3. IFN-$\gamma$ production in culture supernatant of splenocyte stimulated with Con A and CAWS in vitro

Splenocyte ($1\times10^6$) was stimulated by ConA and CAWS in vitro. After 72hr incubation, splenocyte was collected and measured IFN-$\gamma$ concentration by ELISA as described in materials & methods. The results shown represent the mean$\pm$S.D. *, $P<0.05$ and **, $P<0.005$ compared with control (Con A; 1 $\mu$ g/mL). [A]: DBA/2. [B]: C3H/HeN

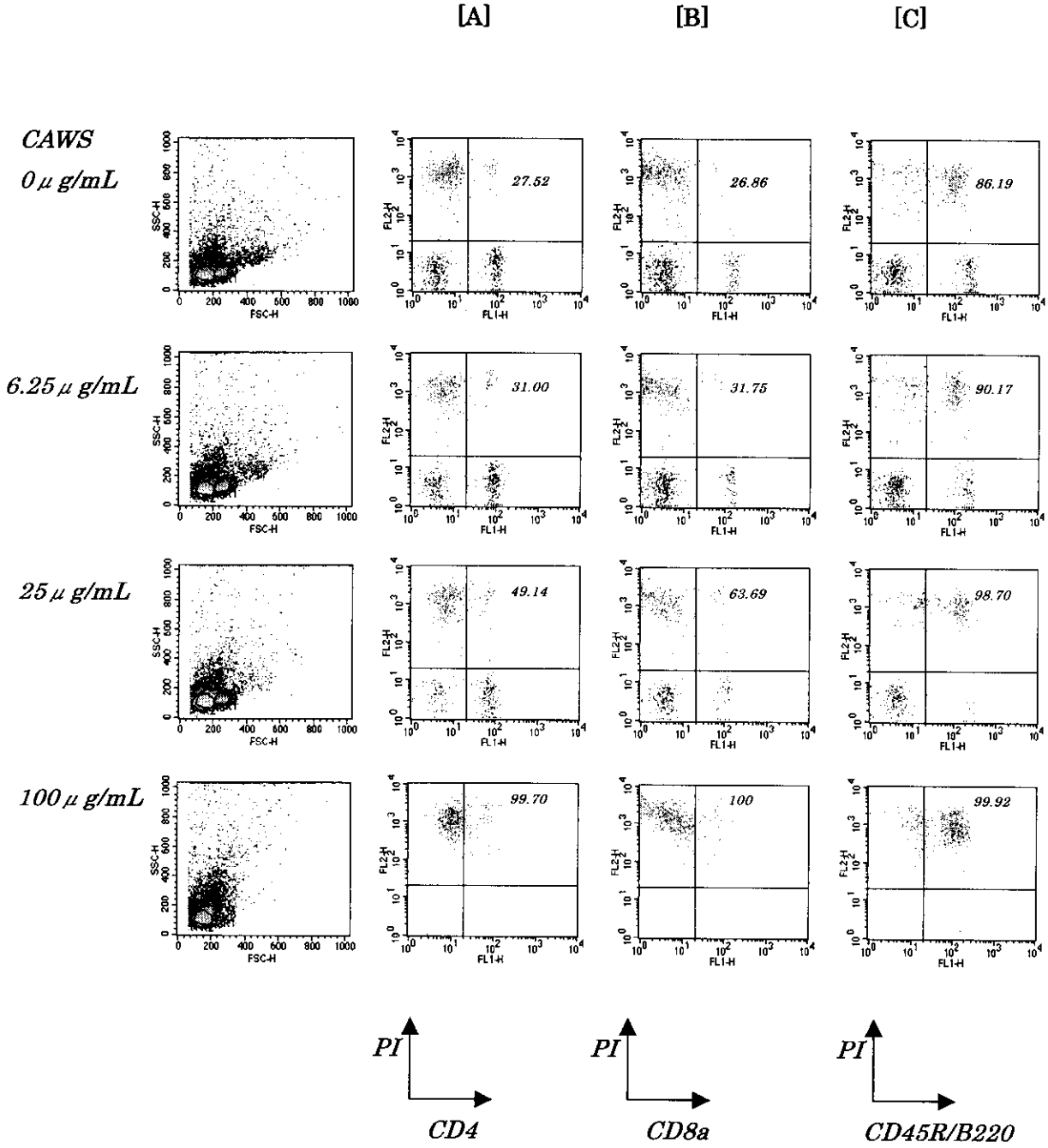


Fig. 4. PI stain of DBA/2 splenocyte stimulated with CAWS in vitro
Splenocyte ($1 \times 10^6$) was stimulated by CAWS in vitro. After 24hr incubation, splenocyte was collected and stained with FITC labeled anti-CD4 (A), CD8α (B) or CD45R/B220 (C) mAb and PI as described in materials & methods. These cells were analyzed by FACS. Numbers represent the ratio of PI-positive cells in surface antigen-positive cells/total surface antigen-positive cells × 100. The data represents one of the results of similar experiments.

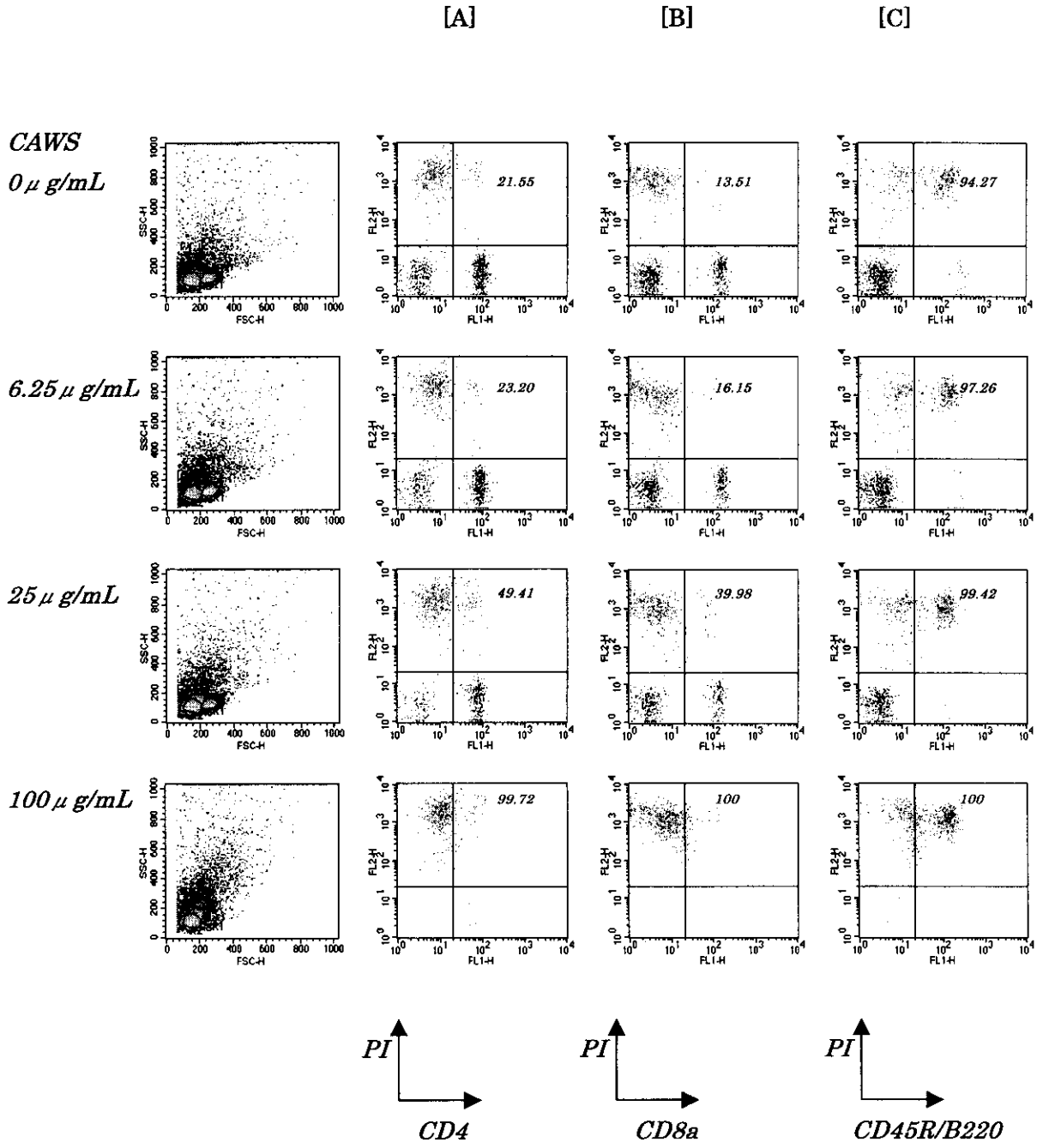


Fig. 5. PI stain of C3H/HeN splenocyte stimulated with CAWS in vitro

Splenocyte ($1 \times 10^6$) was stimulated by CAWS in vitro. After 24hr incubation, splenocyte was collected and stained with FITC labeled anti-CD4 (A), CD8α (B) or CD45R/B220 (C) mAb and PI as described in materials & methods. These cells were analyzed by FACS. Numbers represent the ratio of PI-positive cells in surface antigen-positive cells/total surface antigen-positive cells × 100. The data represents one of the results of similar experiments.

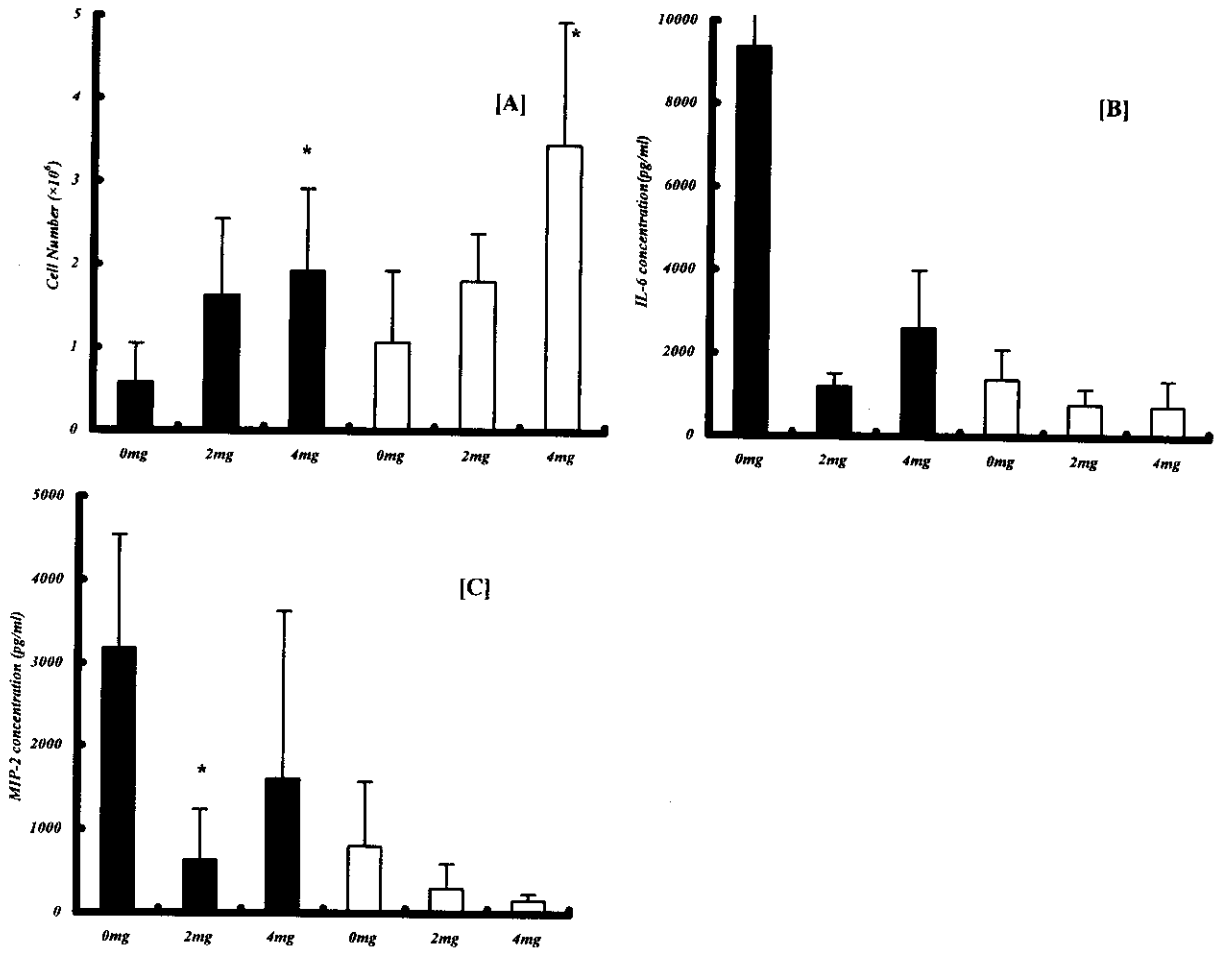


Fig. 6. Cell number and cytokines production of PECs from CAWS intraperitoneally administered mice

CAWS (0, 2 or 4 mg/mouse) was intraperitoneally administered to DBA/2 and C3H/HeN mice for five consecutive days. The PECs were collected from each group of sacrificed mice at 90min after final administration. Total cell number was counted with a hemocytemeter. IL-6 and MIP-2 concentration were measured by ELISA as described in materials & methods. Black and white bars show the results from DBA/2 mice and C3H/HeN mice, respectively. The results shown represent the mean±S.D. *, $P<0.05$ and **, $P<0.005$ compared with control (0mg) by Student's t test. [A]: cell number. [B]: IL-6. [C]: MIP-2.

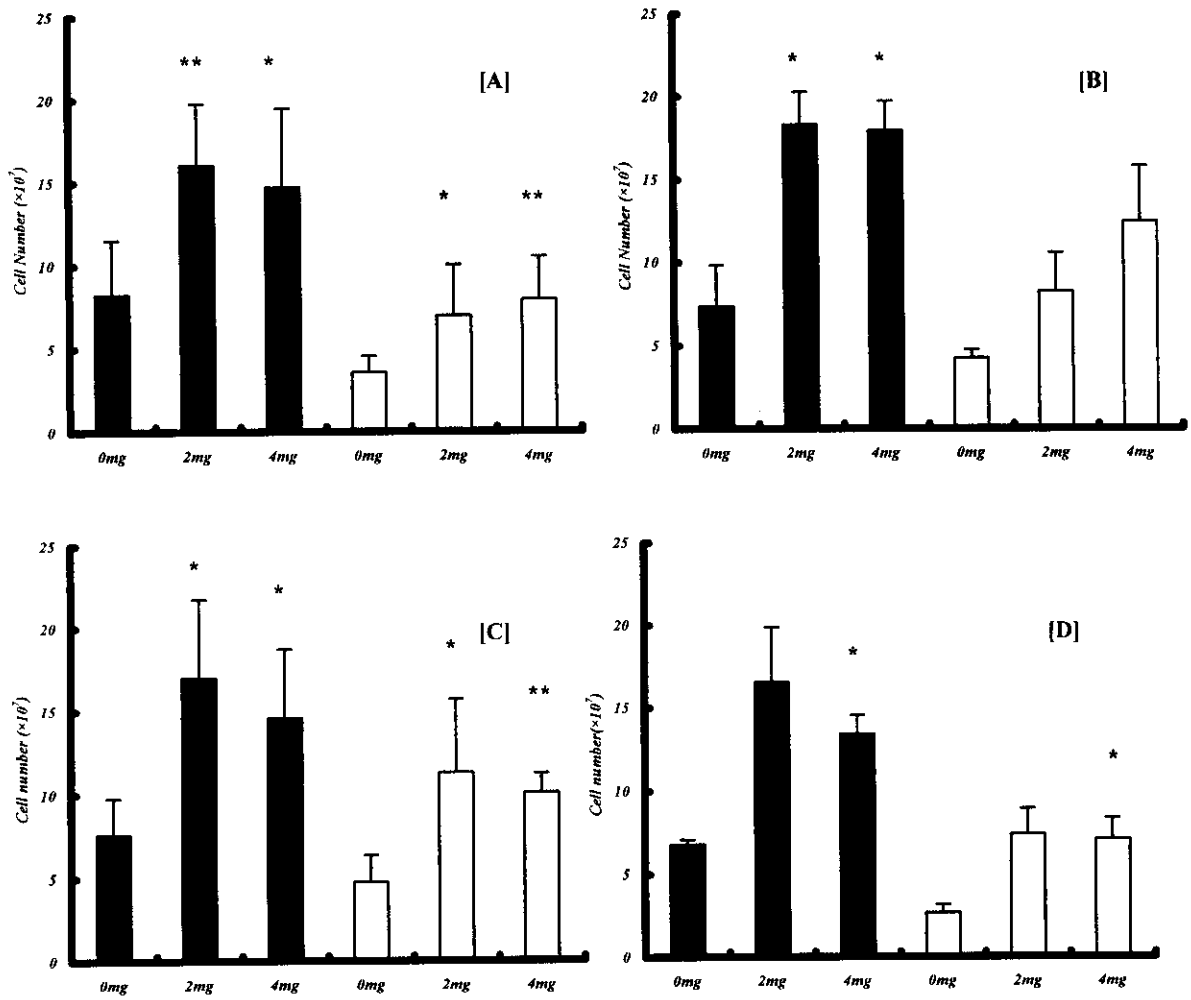


Fig. 7. Cell number of splenocytes from CAWS intraperitoneally administered mice
CAWS (0, 2 or 4 mg/mouse) was intraperitoneally administered to DBA/2 and C3H/HeN mice for five consecutive days. The splenocytes were collected from each group of sacrificed mice at 90min, 1week, 3weeks or 5weeks after final administration. Total cell number was counted with a hemocytemeter. Black and white bars show the results from DBA/2 mice and C3H/HeN mice, respectively. The results shown represent the mean ±S.D. *, $P<0.05$ and **, $P<0.005$ compared with control (0mg) by Student's t test. [A]: 90min after final administration. [B]: 1week after final administration. [C]: 3week after final administration. [D]: 5week after final administration

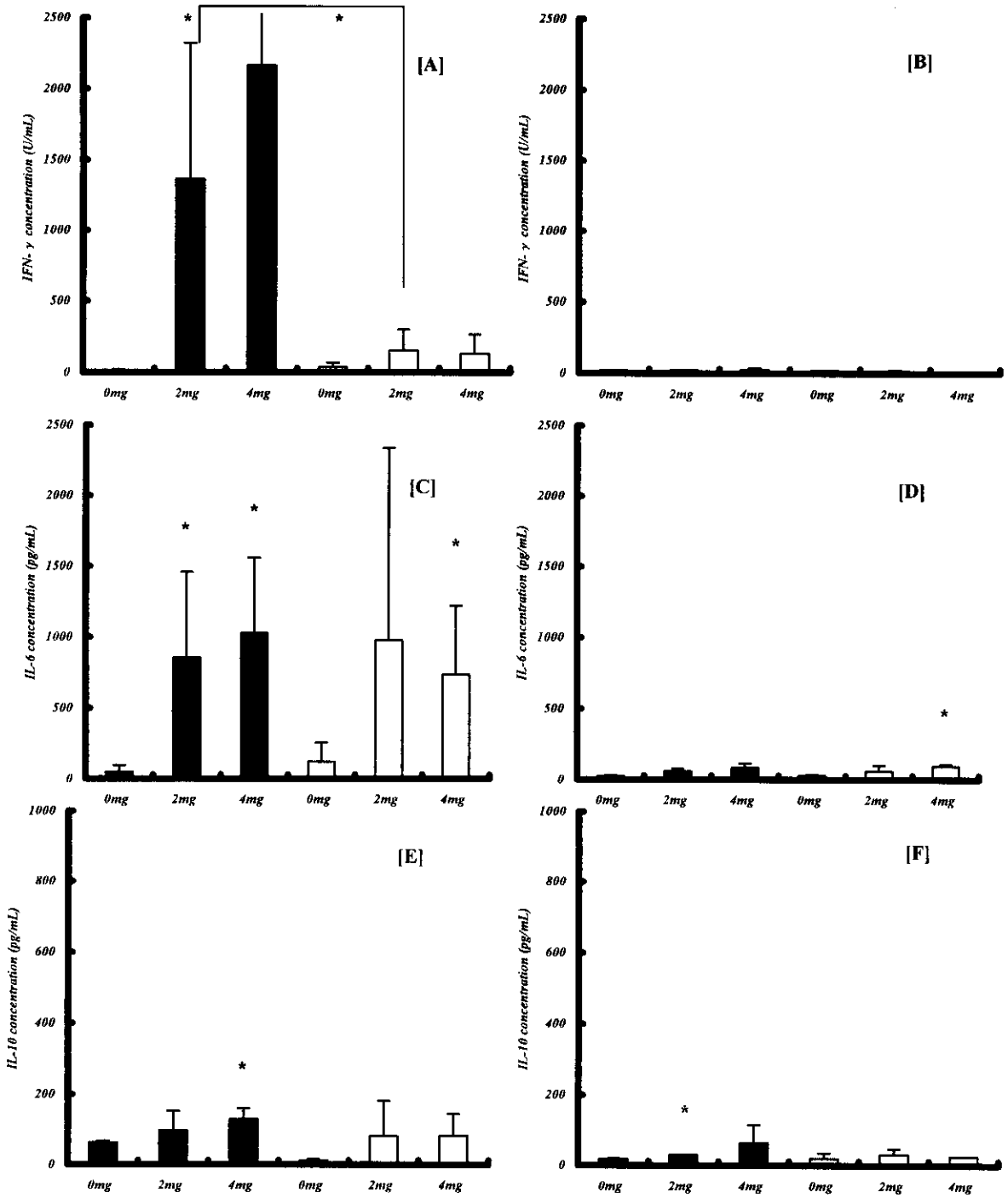


Fig. 8. Cytokines production in culture supernatants of splenocytes from CAWS intraperitoneally administered mice

CAWS (0, 2 or 4 mg/mouse) was intraperitoneally administered to DBA/2 and C3H/HeN mice for five consecutive days. The splenocytes were collected from each group of sacrificed mice at 90min or 1week after final administration. IFN-γ, IL-6 and IL-10 concentration were measured by ELISA as described in materials & methods. Black and white bars show the results from DBA/2 mice and C3H/HeN mice, respectively. The results shown represent the mean±S.D. *, $P<0.05$ and **, $P<0.005$ compared with control (0mg) by Student's t test. [A]: IFN-γ, 90min after final administration. [B]: IFN-γ, 1week after final administration. [C]: IL-6, 90min after final administration. [D]: IL-6, 1week after final administration. [E]: IL-10, 90min after final administration. [F]: IL-10, 1week after final administration.

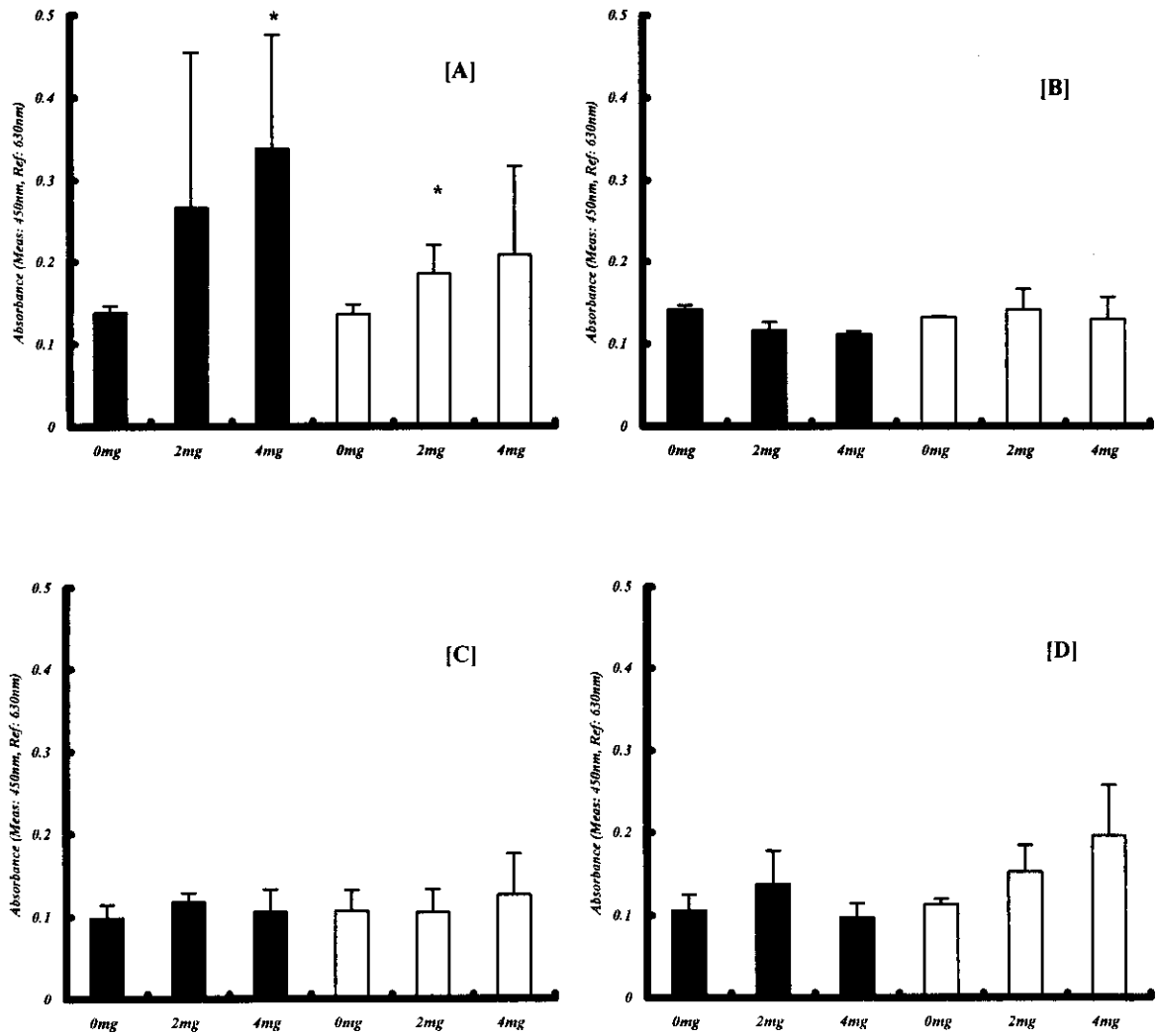


Fig. 9. Myeloperoxidase activity in culture supernatants of splenocytes from CAWS intraperitoneally administered mice

CAWS (0, 2 or 4 mg/mouse) was intraperitoneally administered to DBA/2 and C3H/HeN mice for five consecutive days. The splenocytes were collected from each group of sacrificed mice at 90min, 1week or 3week after final administration. Myeloperoxidase activity was measured as described in materials & methods. Black and white bars show the results from DBA/2 mice and C3H/HeN mice, respectively. The results shown represent the mean±S.D. *, $P<0.05$ and **, $P<0.005$ compared with control (0mg) by Student's t test. [A]: 90min after final administration. [B]: 1week after final administration. [C]: 3week after final administration. [D]: 5week after final administration

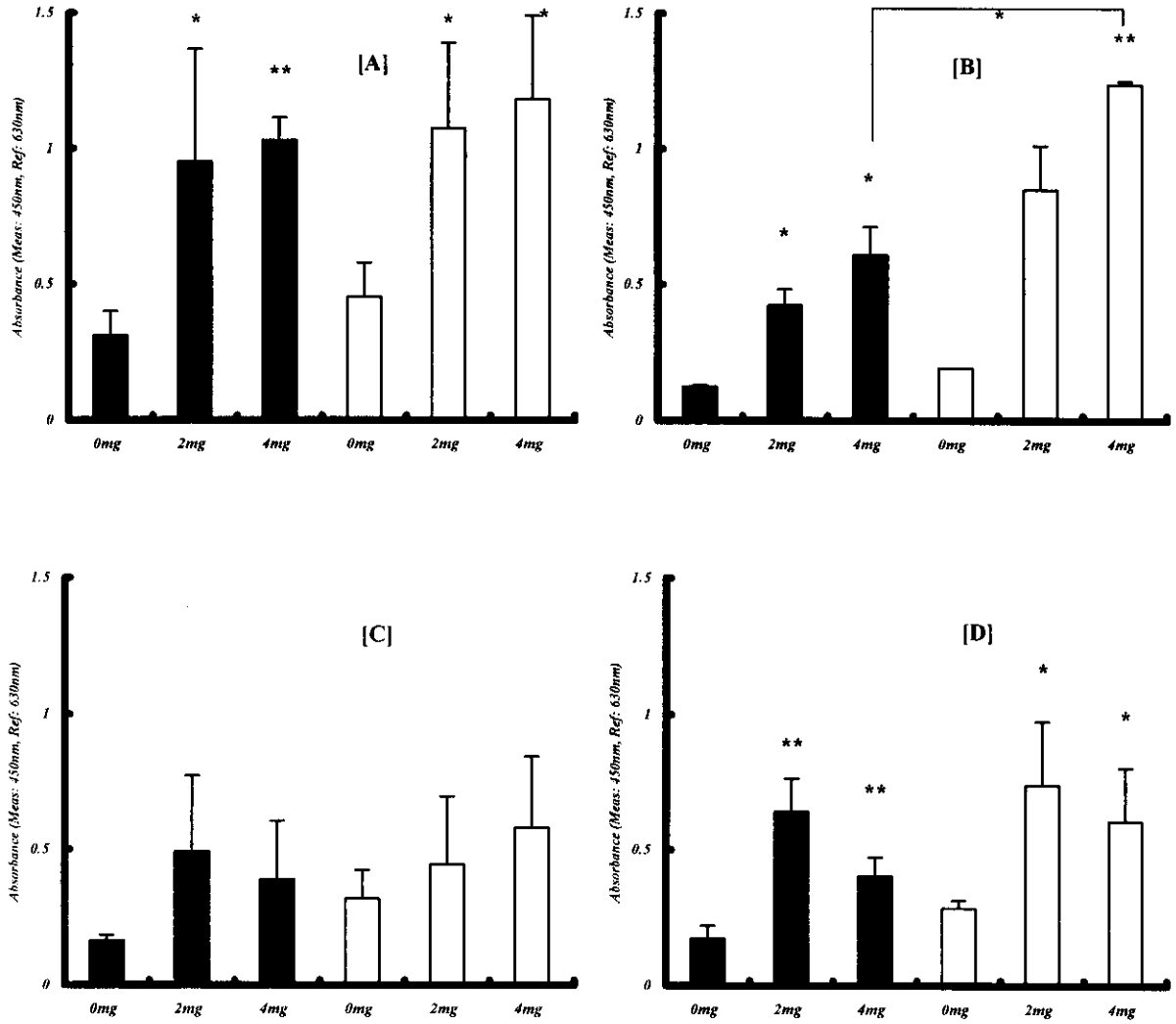


Fig. 10. Myeloperoxidase activity in cell suspensions of splenocytes from CAWS intraperitoneally administered mice

CAWS (0, 2 or 4 mg/mouse) was intraperitoneally administered to DBA/2 and C3H/HeN mice for five consecutive days. The splenocytes were collected from each group of sacrificed mice at 90min, 1week or 3weeks after final administration. Myeloperoxidase activities were measured as described in materials & methods. Black and white bars show the results from DBA/2 mice and C3H/HeN mice, respectively. The results shown represent the mean± S.D. *, $P<0.05$ and **, $P<0.005$ compared with control (0mg) by Student's t test. [A]: 90min after final administration. [B]: 1week after final administration. [C]: 3week after final administration. [D]: 5week after final administration.

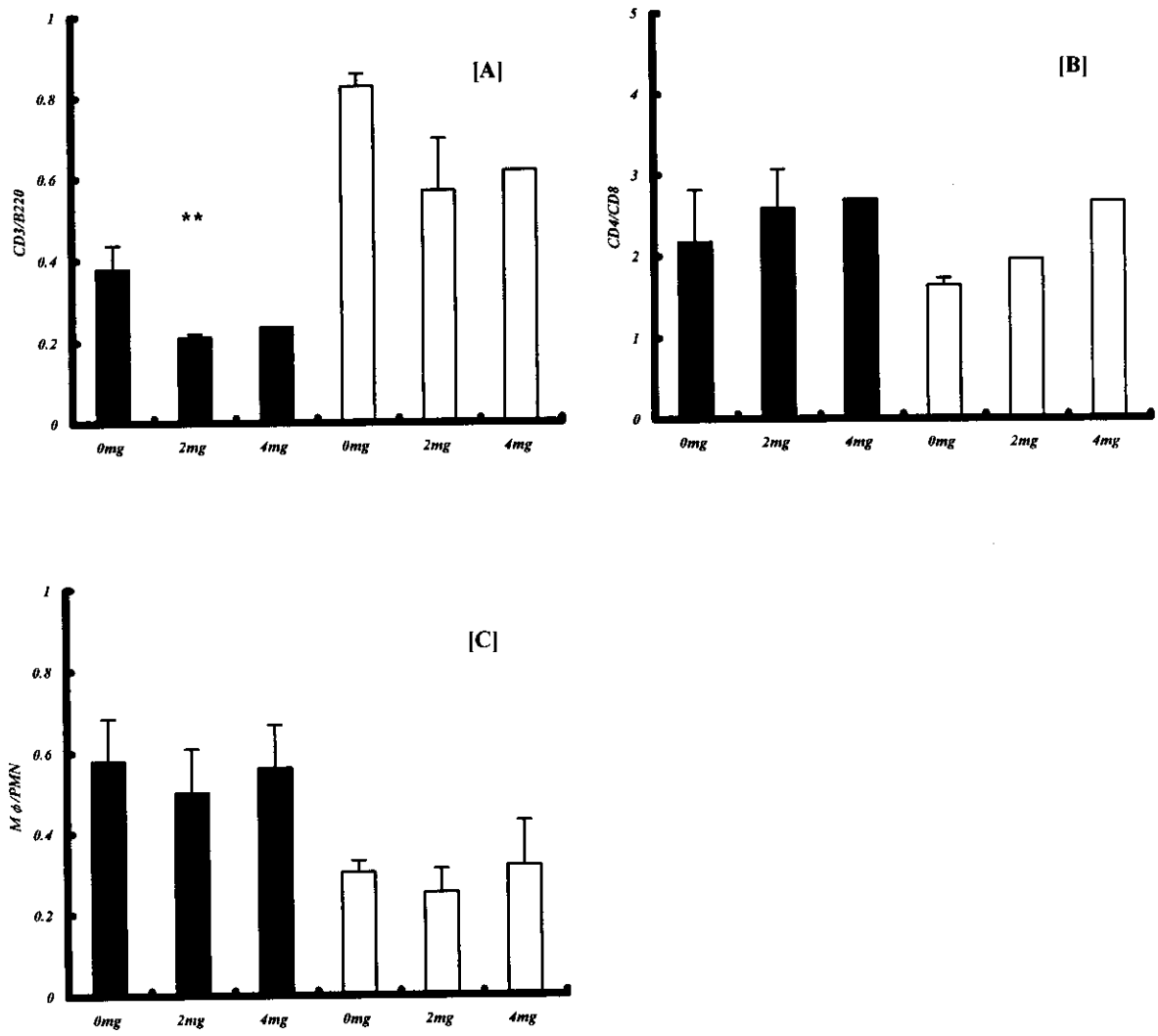


Fig.11. Change of cell population in splenocytes from CAWS intraperitoneally administered mice

CAWS (0, 2 or 4 mg/mouse) was intraperitoneally administered to DBA/2 and C3H/HeN mice for five consecutive days. The splenocytes were collected from each group of sacrificed mice at 90min after final administration. Splenocytes population was measured by flow cytemetory described in materials & methods. Black and white bars show the results from DBA/2 mice and C3H/HeN mice, respectively. The results shown represent the mean±S.D. *, $P<0.05$ and **, $P<0.005$ compared with control (0mg) by Student's t test. [A]: CD3/B220. [B]: CD4/CD8 [C]: Gr-1/Mac-1

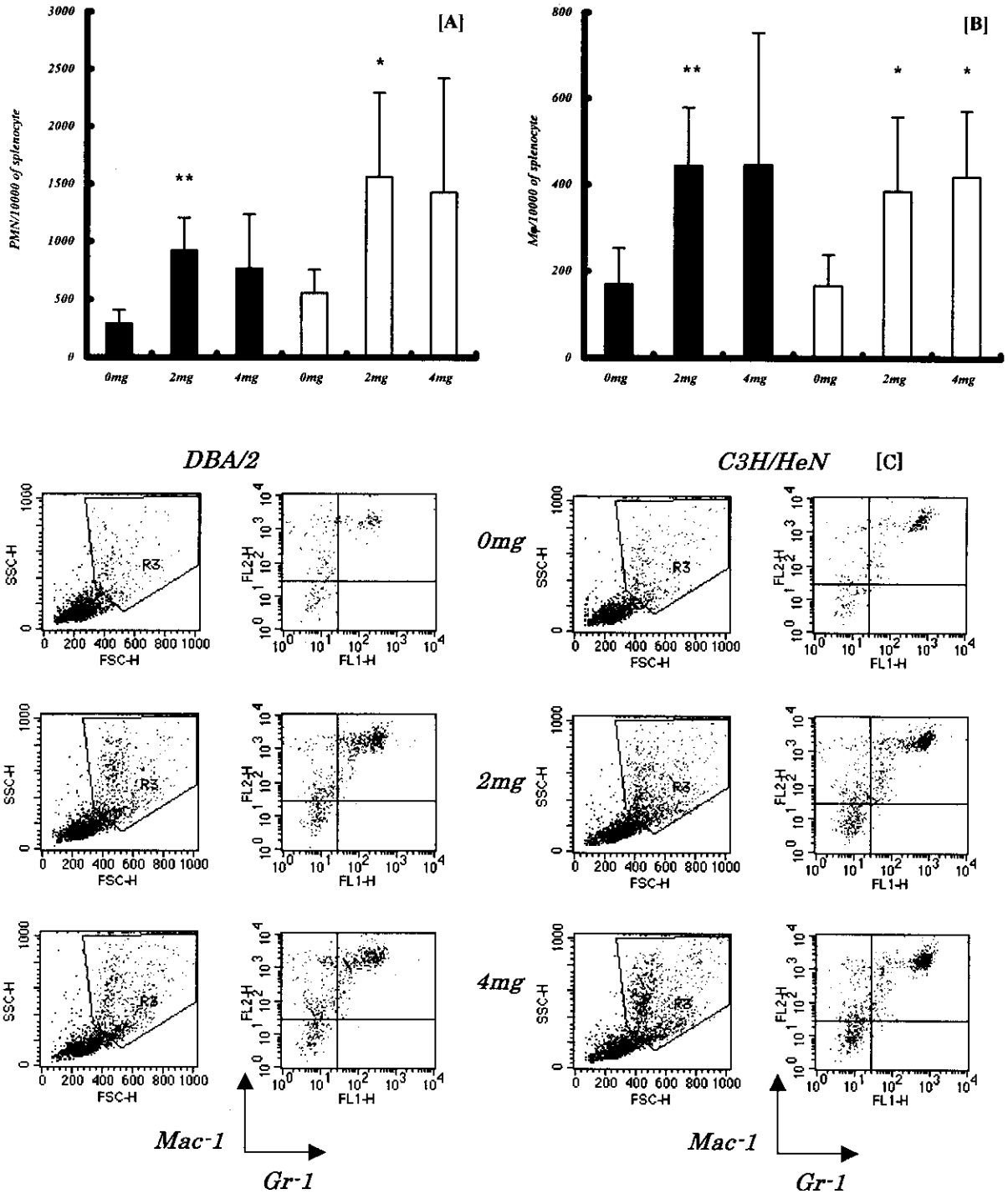


Fig.12. Ratio of granulocyte and macrophage in splenocytes from CAWS intraperitoneally administered mice

CAWS (0, 2 or 4 mg/mouse) was intraperitoneally administered to DBA/2 and C3H/HeN mice for five consecutive days. The splenocytes were collected from each group of sacrificed mice at 90min after final administration. Cell count of Granulocyte and Macrophage were measured by flow cytemetory described in materials & methods. Black and white bars show the results from DBA/2 mice and C3H/HeN mice, respectively. The results shown represent the mean ±S.D. *, $P<0.05$ and **, $P<0.005$ compared with control (0mg) by Student's t test. [A]: PMN. [B]: Mφ. [C]: displayed with scatter mode and FL-1/FL-2

厚生科学研究費補助金（高度先端医療研究事業）
分担研究報告書

# MPOに対する単クローン抗体の作製

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研究要旨： MPO－ANCAをモデルとして、自己免疫疾患の治療に有効な単クローナル抗体（mAb）の作製を担当した。今年度は、市販MPOの完全分子を用いてそれらに特異的なmAbの確立を試みた。更に、mAbを臨床に適用する際の国際的規制（WHO, FDA, ICH, CPMP）内容を整理し、今後の臨床応用に備えることにした。

A．研究目的

好中球の細胞質自己抗体 MPO-ANCA と MPO との免疫複合体は、病変部で好中球を活性化して種々の組織機能障害をもたらす可能性のあることが示唆されている。そこで、MPO-ANCAをモデルとして、自己免疫疾患の治療に有効な単クローナル抗体（mAb）の作成を担当した。今年度は、市販MPOの完全分子を用いてそれらを臨床に適用する場合の国際的規制（MHO, FDA, ICH, CPMP）内容を整理し、今後の臨床応用に備えることにした。

B．研究方法

市販ヒト型ＭＰＯをＢＡＬＢ/ｃマウスに所定の方法で免疫し、一定期間後に脾臓を取り出し、SP2/0 ミエローマ細胞と PEG4000 を用いて融合させた。 HAT 培地で選択したハイブリドーマをELISAでスクリーニングし、MPO 陽性細胞を限界希釈法で選択した。MPOに特異的かどうかのチェックは、Mycoplasma spp.を抗原にしたELISAとMPOを抗原にしたWestern blottingで行った。

２．単クローナル抗体（mAb）の臨床応用

本研究事業中におけるmAbの臨床応用を考え、mAb の臨床応用に関する国際的指針（WHO Guideline, FDA/Federal Register, EU/CPMP Guidance, ICH/Q5A Guideline）における要件を整理した。

（倫理面への配慮）現時点ではなし。

C．研究結果

１．MPOに対する単クローン抗体の作製

ELISA で陽性細胞が幾つか検出された。これらの特異性について現在検討中である。本研究開始後、約半年間、ハイブリドーマの形成率が悪く、研究遂行上困難を極めた。研究的には無駄な時間ではあったが、問題解決は今後の研究発展に大きな収穫となった。

２．単クローナル抗体（mAb）の臨床応用

医薬品には前臨床段階から種々の規制がかかってくる。とりわけ、バイテク製剤や生物学的製剤は、使用原料や製法等の特性により、未知のウイルスや異物（蛋白質、核酸、エンドトキシン、マイコプラズマ等）が混入する危険性が常にある。そのため、mAb 産生細胞（MCB：Master cell bank、WCB：Working cell bank、EPC：End-of-production cells）や、mAb（未精製バルク、精製バルク、最終製品）については、種々の段階で各種試験が要求さ

れている。とりわけ、ウイルスの不活化、除去工程については、十分なバリデーションデータが要求される。更にmAbの臨床応用にはGLP, GMP, GCP等の幅広い知識も必要となる。

D．考察

本研究事業は、「抗体作製班」と「評価研究および臨床研究班」から構成されており、分担研究者は、前者に所属しmAb産生ハイブリドーマの確立を目指してきた。細胞融合系は順調に機能しているので、MPOに特異的なmAb産生細胞の確立は難しいものではない。それをいかにモデル動物等を用いて臨床的観点から評価するかが課題である。第二年度は、mAbを作製するにしてももっと臨床的な観点からターゲット（抗原エピトープ）を絞る必要があるかもしれない。MAbの臨床応用に関しての法的規制は掌握できたので、機能性をもったmAbが確立されたら、動物実験（前臨床試験）から臨床治験への道のりは開けた。

E．結論

MPOに特異的なmAbがいくつか確立されつつある。それらの機能性については、第二年度に追求することになる。MAb（マウス型又はヒト型mAb)の臨床応用についも国際的指針内容に基づき、その方向性は開けた（添付資料参照)。

F．健康危険情報

なし

G．研究発表

本研究事業内容に関する研究発表はなし。

H．知的所有権の取得状況

1．特許取得　　なし

2．実用新案登録　なし

3．その他　　なし

# 単クローン抗体の臨床応用に関するWHOガイドライン

国立感染症研究所
佐々木次雄

# Guidelines for Monoclonal Antibodies

- Guidelines for assuring the quality of monoclonal antibodies for use in humans. WHO Tec. Rep. Ser., No. 822, 1992.
- Points to consider in the manufacture and testing of monoclonal antibody products for human use. Federal Register, 62 (40), 1997.
- Guidance for industry monoclonal antibodies used as reagents in drug manufacturing. FDA, 1999.

## Seed Lot System

- **Master cell bank: homogeneous cell suspension derived from the original cell line.**
- **Working cell bank: a quantity of cells of uniform composition, derived from one or more containers of the master cell bank and stored frozen in the vapor phase of liquid nitrogen.**

## Methods of Production

- **Batch system: the cell-culture fluid (s) from the production vessel (s) is/are harvested on a single occasion.**
- **Continuous cell-culture system: the cell-culture fluid (s) from the vessel (s) used for the last passage is/are harvested on multiple occasions.**
- **In both system, the maximum number of passages or population doublings of cells of the WCB is approved by the licensing authority.**

# Production Stages

- Single harvest: filtrate obtained from one batch of cell cultures seeded.
- Bulk harvest: homogeneous pool of single harvest.
- Purified bulk: product after purification of a bulk harvest is completed.
- Final bulk: homogeneous preparation present in a single container.
- Final product: finished product that is formulated and dispended into final, sealed containers.
- Final lot: a collection of sealed final containers.

# General Manufacturing Requirements

- Good manufacturing practices for pharmaceutical products.
- Good manufacturing practices for biological products.
- Requirements for Biological Substances No. 27 (Requirements for the Collection, Processing, and Quality Control of Blood Components, and Plasma Derivatives).

## General Procedures for Generating Hybridomas

### Material used for immunization

- **Materials used for immunization: the material used for the generation of immune lymphocytes should be defined. If the immunization is derived from a human source, relevant clinical data on the donor should be recorded.**

## General Procedures for Generating Hybridomas

### Immune parenteral cells

- **For murine monoclonal antibodies, information on the animal strain shall be provided, including its specific-pathogen-free (SPF) status.**
- **For human monoclonal antibodies, the donations employed should be screened for potential viral contamination.**

## Tests for Master Cell and Working Cell Banks

- Identity test for the product
- Tests for bacteria, fungi and mycoplasmas
- Test for viruses

## Production from Mouse Ascitic Fluid

- If substances other than pristane are used to pretreat mice to facilitate the growth of hybridomas, they shall be approved by the national control authority.
- The mice shall come from SPF-monitored colonies and in particular shall be free from the viruses in List 1.

## Removing of Contaminated Cellular DNA

- DNA quantities should be less than 100 pg per single human dose.
- Validation studies for the removal of cellular DNA should be performed by spiking source materials with known large amounts of representative DNA.

## Production Control

### Purified Bulk

- Test for components other than those serected by the cell line or the hybridoma
- Test for residual DNA
- Test for contaminating viruses

# Production Control
## Final Bulk

- Test for identity
- Test for bacteria and fungi
- Test for purity
  - Test for distribution of molecular size
  - Isoelectric focusing
  - SDS-PAGE
  - Test for protein content

# Production Control
## Final Product

- Test for identity
- Test for potency
- Test for pyrogenicity
- Test for bacteria and fungi
- Test for abnormal toxicity
- Test for protein content
- Test for residual moisture
- Test for pH
- Test for preservative
- Inspection of final containers

## Possible Viral Contaminants in Hybridomas of Murine Origin

- There is evidence of capacity for infecting humans or primates.

- Hantaan virus, Lymphocyte choriomeningitis virus, Rat rotavirus, Reovirus type 3, Sendai virus

## Possible Viral Contaminants in Hybridomas of Murine Origin

- There is no evidence of capacity for infecting humans

- Ectromelia virus, K virus, Kiham rat virus, Lactic dehydrogenase virus, Minute virus of mice, Mouse adenovirus, Mouse cytomegalovirus, Mouse rotavirus, Murine hepatitis virus, Murine poliovirus, Pneumonia virus of mice, Poliomavirus muris 1, Rat coronavirus, Retroviruses, Sialodacryoadenitis virus, Thymc virus, Toolan virus