면역독성 biomarker의 인체유해성평가 적용

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면역계는 화학물질에 의한 독성에 비교적 안정할 것으로 여겨지지만 실제로는 화학물질에 의한 독성에 매우 민감하다. 면역계는 매우 다양한 세포들이 서로 유기적인 협동하에 생체를 방어하고, 지속적으로 증식 및 분화과정을 거쳐 특수한 기능을 담당하게 되므로 세포의 주기에 영향을 주거나 또는 cytokine이나 세포표면 항원과 같은 단백질들의 합성과정에 영향을 줄 수 있는 물질들은 면역계에 심각한 영향을 줄 수 있다. 또한 면역세포 자체는 약물대사능이 거의 없지만 다른 장기에서 활성화된 대사체가 영향을 줄 수 있다. 그리고 신경계, 내분비계 등도 2차적으로 면역기구의 조절에 역할을 담당하므로 화학물질들 중에서 신경계, 내분비계 및 간에 영향을 줄 수 있는 물질은 면역계에도 영향을 미쳐 면역억제나 면역항진과 같은 독성이 유발될 수 있다. 면역계가 화학물질에 의한 독성 유발에 있어서 중요한 목표장기(target organ)가 될 수 있다는 사실은 1970년대 초 미국의 미시간주에서 발생한 polybrominated biphenyl이 오염된 낙농제품의 섭취에 의한 면역계 질환이 보고되고 부터이며, 이후 미국에서는 화학물질에 의한 면역계 이상을 판단할 수 있는 각종 시험법들의 개발에 박차를 가하게 되었다. 또한 면역억제제를 복용하고 있는 환자들에 있어서 감염성 질환의 발생과 암발생율이 정상인에 비해 현저히 높다는 것을 인지하면서 이전에는 별로 독성연구에 관심이 기울여지지 않았던 면역계에 대하여 독성 평가가 필요함을 인식하게 되었다. 그래서 이후 미국의 National Toxicology Program(NTP)을 주축으로 화학물질에 의한 면역독성 평가에 관한 시험기준을 마련하게 되었다. 그러나 기존의 독성평가 시험과는 달리 면역계를 구성하는 세포와 각 세포의 기능이 매우 다양하므로, 단일 항목의 시험만으로는 면역계에 대한 독성 평가를 제대로 할 수 없는 관계로 여러가지 시험항목들로 구성된 Tier system을 도입하여 상당히 많은 종류의 시험법들을 사용하도록 하고 있다. 화학물질에 의한 인체유해성 평가는 제한된 실험실에서 이루어지고 있으며, 사용되는 면역독성 biomarker들도 제한적이다. 본 연구에서는 현재 실험동물에서 사용되고 있는 면역독성 지표들에 대한 내용을 국내에서 1995년에 문제가 되었던 2-bromopropane과 발암물질인 ethyl carbamate에 대한 연구결과를 토대로 발표하고자 하며, in vitro 시험계에서 대사능이 거의 없는 비장세포 배양을 이용하여 대사후 면역독성을 나타내는 독성물질의 검출에 관한 연구기법을 소개하고자 한다.

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면역독성 **biomarker**의 인체유해성 평가 적용

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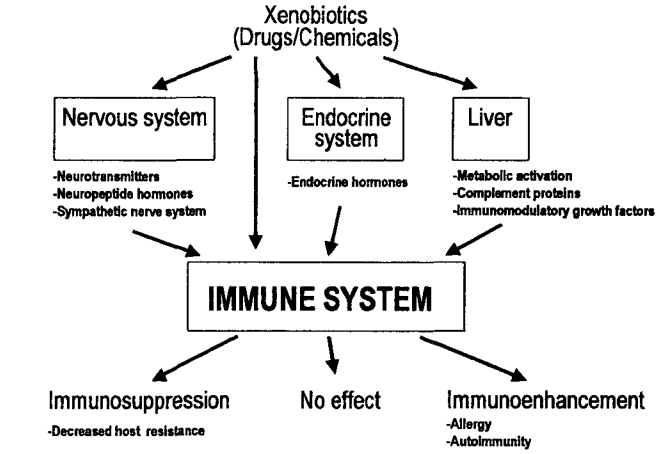
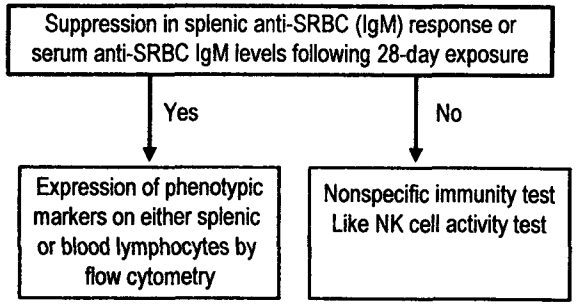
Cellular components of immune system

Origin	Common progenitors	Specialized progenitors	Differentiated progeny
Stem cells	Myeloid	Reticulocyte	Erythrocyte
		Megakaryocyte	Platelet
		Promonocyte	Monocyte/Macrophage
		Granulocyte	Neutrophil
			Eosinophil
			Basophil
			Mast cell
	Lymphoid	B-Cell	Plasma cell
		T-Cell	T helper/inducer
			T cytotoxic
			T suppressor
		Null cell	Natural killer (NK)

Effects of xenobiotics on immunity & potential outcomes



Principles of immunotoxicity guidelines by US EPA



US NTP guidelines for detecting immune alterations
Tier I (screen) tests

Parameters	Procedures
Immunopathology	Hematology-complete blood count & differential
	Weights-body, spleen, thymus, kidney, liver
	Cellularity-spleen
	Histology-spleen, thymus, lymph node
Humoral-mediated immunity	IgM antibody plaque-forming cells to T-dependent antigen (SRBC)
	LPS mitogen response
Cellular-mediated immunity	Lymphocyte blastogenesis to mitogen (Con A)
	Mixed leukocyte response against allogeneic leukocytes
Nonspecific immunity	Natural killer (NK) cell activity

US NTP guidelines for detecting immune alterations
Tier II (comprehensive) tests

Parameters	Procedures
Immunopathology	Quantitation of splenic B & T cell numbers
Humoral-mediated immunity	Enumeration of IgG antibody response to SRBCs
Cellular-mediated immunity	Cytotoxic T lymphocyte (CTL) cytolysis
	Delayed hypersensitivity response (DHR)
Nonspecific immunity	Quantitation of resident peritoneal macrophages
	Phagocytic ability of macrophages
Host resistance challenge models (endpoints)	Syngeneic tumor cells -PYB6 sarcoma (tumor incidence) -B16F10 melanoma (lung burden)
	Bacterial models -*Listeria monocytogenes* (mortality) -*Streptococcus* species (mortality)
	Viral models -Influenza (mortality)
	Parasite models -*Plasmodium yoelii* (parasitemia)

Korean MHW's guidelines for detecting immune alterations

Parameters	Procedures
Antigenicity	-Anaphylactic shock reaction -Passive cutaneous anaphylaxis reaction -Maximization test
Cellular immunity	-Lymphoproliferation assay with Con A, PHA, or specific antigens -Mixed leukocyte response -Delayed-type hypersensitivity to ovalbumin, tuberculin, or *Listeria*
Humoral immunity	-Splenic plaque-forming cell assay -Blood concentration of antibodies to T-cell dependent antigen -Blood concentration of antibodies to T-cell independent antigen, lipopolysaccharide -Lymphoproliferation with lipopolysaccharide
Macrophage functions	-Phagocytic activity to Listeria monocytogenes -Cytotoxic activity to YAC-1 cells in mice & K562 cells in human -Carbon clearance test
Natural killer cell function	-Cytotoxicity to YAC-1 cells in mice & K562 cells in human

2-Bromopropane (2BP)

- Major component of the mixture of SPG-6AR & Solvent 5200 which is a substitute of chlorofluorocarbon in electronic factory
- In 1995, a health problem by an occupational exposure to 2BP has been a social issue in Korea: female workers had amenorrhea & male workers had oligospermia.
- Following the accident, toxic potential of 2BP has extensively been investigated.
- 2BP caused reproductive toxicities in both male & female rats.
- 2BP induced mutagenicity in TA 100 & TA 1535 strains with negative results in the chromosomal aberration test in CHL cells and the micronucleus test in the bone marrow of rats.
- In 1999, the occupational exposure level for 2BP has firstly been established in Korea at 1 ppm.
- 2BP caused a significant decrease in the number of white blood cells including lymphocytes

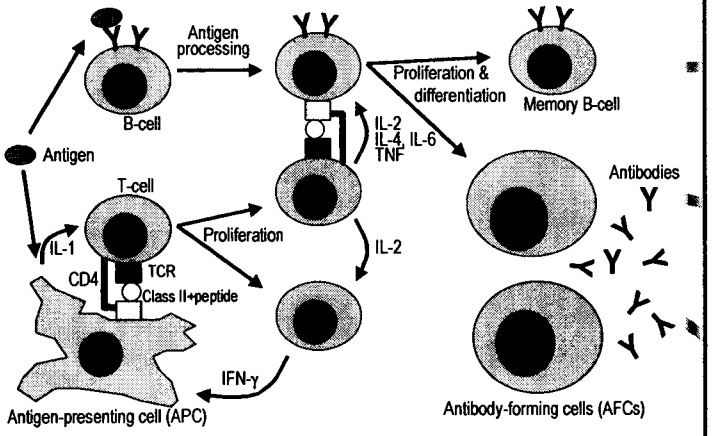
Objectives

- To determine the possible immunotoxic potential of 2BP in male Sprague Dawley rats
- To determine the cellular target in 2BP-induced immunosuppression by flow cytometry
- To determine a possibility of adduct formation with DNA

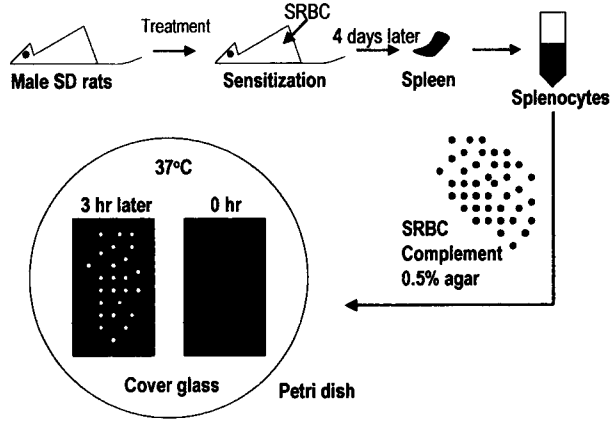
Methods

- Animals: male Sprague-Dawley rats
- Treatment: 2BP in corn oil at 0, 100, 330 or 1000 mg/kg orally for 28 consecutive days
- Hematology & serum clinical chemistry
- Antibody response to sheep red blood cells (SRBCs)
- Flow cytometry of splenic & thymic cell suspensions

Cellular interactions in antibody response



In vivo antibody response to SRBCs



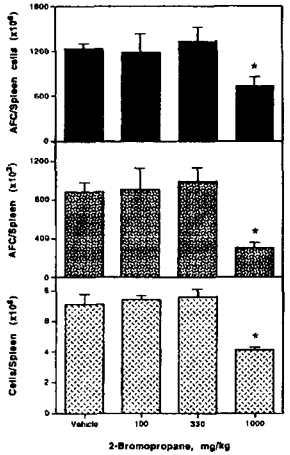
Effects of 2BP on body, spleen & thymus weights

[redacted]

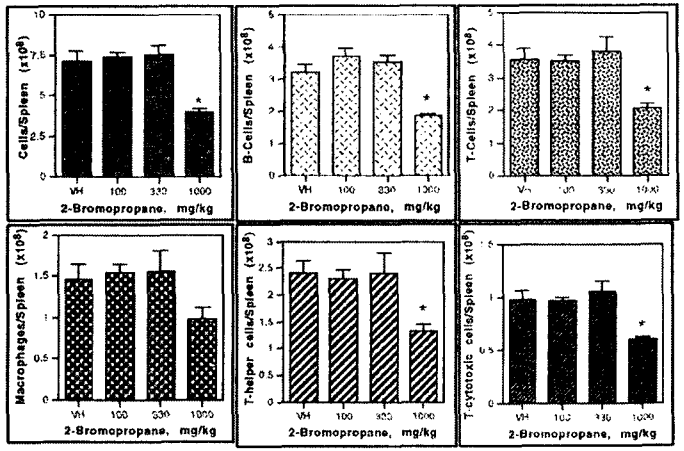
Effects of 2BP on hematological parameters

[redacted]

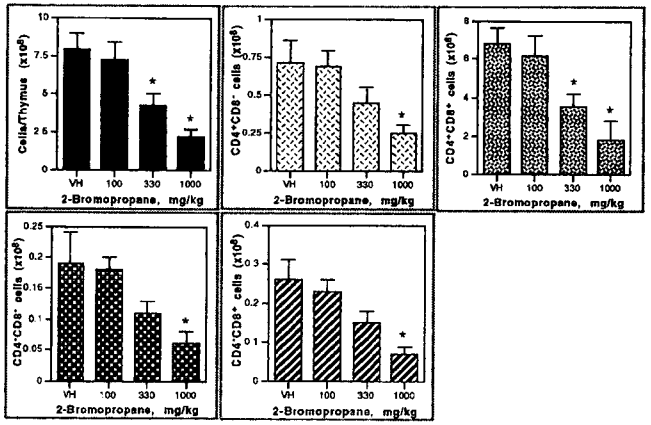
Effects of 2BP on antibody responses to SRBCs



Flow cytometry: Spleen

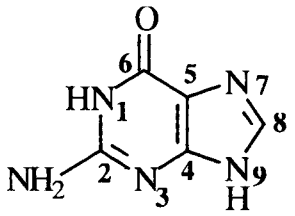


Flow cytometry: Thymus



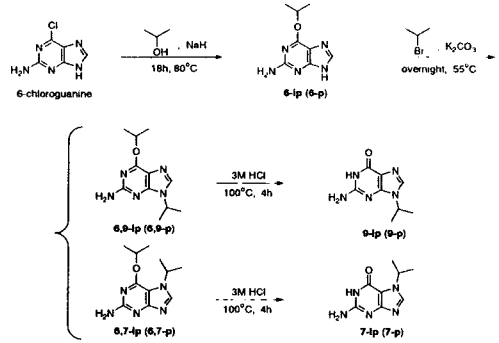
Current studies on mechanism of toxic action: A possibility of adduct formation with DNA

- 2BP induced mutagenicity in TA 100 & TA 1535 strains of *Salmonella typhimurium*.
- An induction of micronuclei formation was reported in mouse embryo after maternal treatment with 2BP.



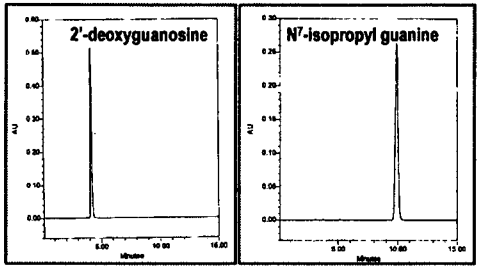
Guanine

Synthetic scheme of N^7-isopropyl guanine

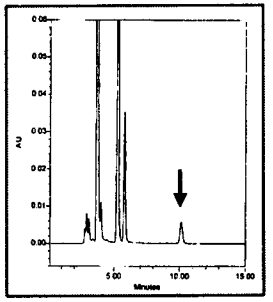


Instead of 2-propanol, using 1-propanol in the first step, and 1-bromopropane in the second step 6-p, 6,9-p, 6,7-p, 9-p, 7-p were formed in the same method as described above

HPLC chromatograms of 2'-deoxyguanosine & N^7-isopropyl guanine

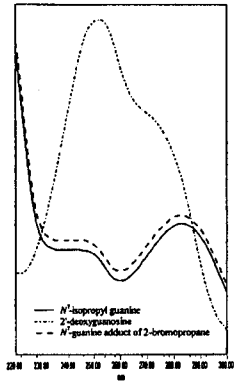


HPLC chromatograms of N^7-isopropyl guanine produced from a reaction of 2-bromopropane with:



2'-deoxyguanosine

UV spectra of N^7-isopropyl guanine, 2'-deoxyguanosine & N^7-guanine adduct of 2-bromopropane



Conclusion (I)

- 2BP could suppress the T-dependent antibody response to SRBCs when treated for 28 consecutive days.
- The flow cytometry indicated that 2BP might be a nonspecific immunotoxicant.
- 2BP could form a guanine adduct in a physiological condition in vitro.

Ethyl carbamate (Urethane)

- Used in human medicine as an anti-neoplastic agent.
- An anaesthetic for laboratory animals
- Can be formed by a reaction of ethanol with carbamyl phosphate in fermented foods & alcohol beverages.
- Present at 0.17 mg/l in orange juice, 2.6 mg/l in white wine and 310~375 ng/g in cigarette.
- Carcinogenic in mice, rats & hamsters by oral, inhalation, subcutaneous or intraperitoneal routes producing lung tumors, lymphomas, hepatomas, melanomas & vascular tumors.
- Immunotoxic potential: decreases in bone marrow precursor cells, host resistance to B16F10 melanoma tumor & T-dependent antibody response to SRBCs *in vivo*.
- Requires metabolic activation by P450 2E1 for mutagenicity.

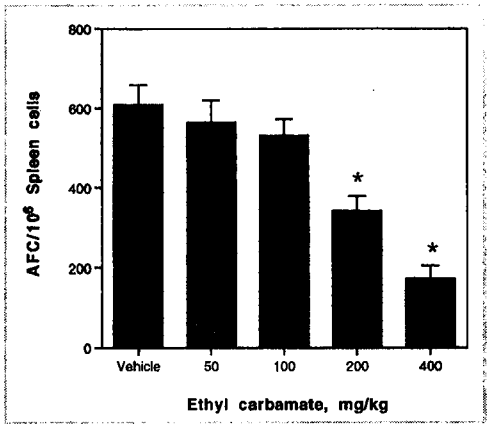
Objectives

- To determine whether ethyl carbamate requires metabolic activation for its immunotoxicity
- To determine a possible role of corticosterone in ethyl carbamate-induced immunosuppression

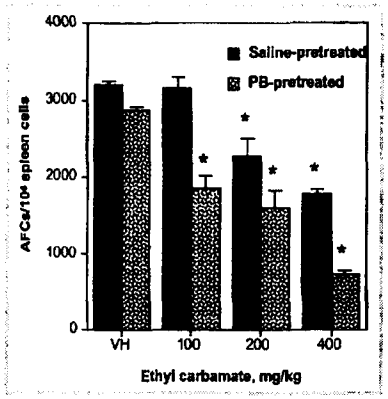
Methods

- Animals: female BALB/c mice
- Treatment:
  - P450 inducer: phenobarbital
  - P450 inhibitor: aminoacetonitrile
  - Esterase inhibitor: diazinon
- Adrenalectomy
- Antibody response to sheep red blood cells (SRBCs)
- Serum corticosterone level by RIA
- Flow cytometry for splenic cell subpopulation

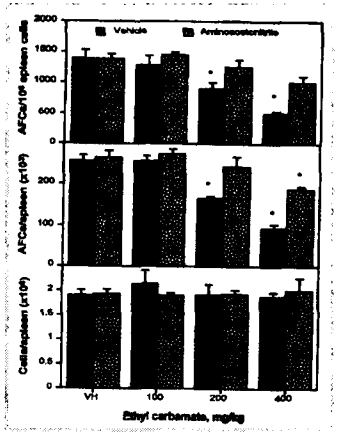
Effects of ethyl carbamate on antibody response to SRBCs



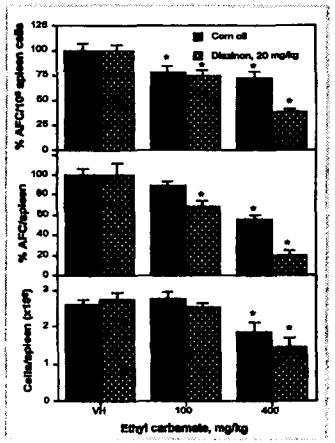
Effects of P450 induction on ethyl carbamate-induced suppression of antibody response



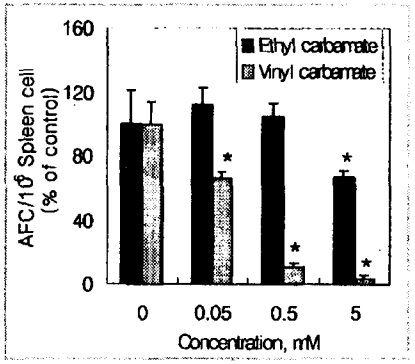
Effects of P450 inhibition on ethyl carbamate-induced suppression of antibody response



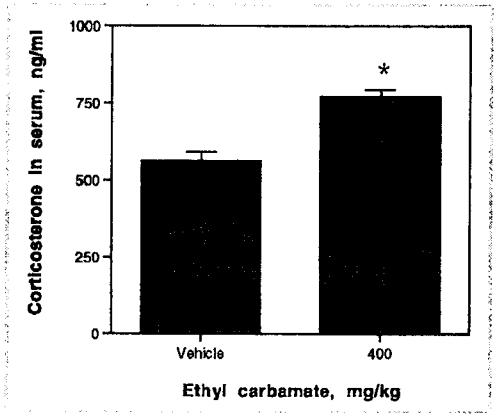
Effects of esterase inhibition on ethyl carbamate-induced suppression of antibody response



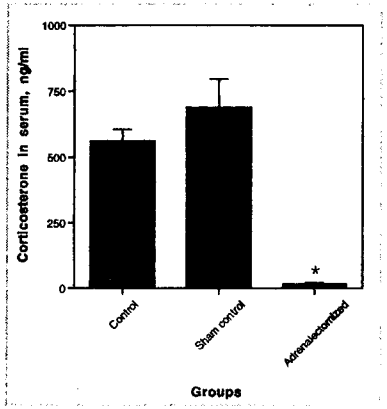
Effects of ethyl carbamate & vinyl carbamate on in vitro antibody response



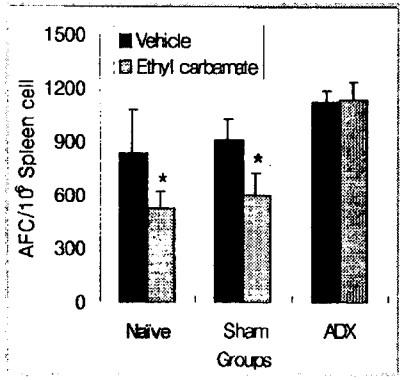
Effects of ethyl carbamate on serum corticosterone



Effects of adrenalectomy on serum corticosterone



Effects of adrenalectomy on ethyl carbamate-induced suppression of antibody response



Conclusion (II)

- Ethyl carbamate requires metabolic activation by cytochrome P450s to suppress the T-dependent antibody response to SRBCs.
- Adrenal hormone(s) might have a role in ethyl carbamate-induced immunosuppression.

Role of metabolic activation in toxicology
In vitro studies. 1.

- Splenocytes can produce antibody response to certain antigen in cultures.
- Splenocytes have a limited capacity for metabolic activation.
- This problem can be overcome by incubation of splenocytes with metabolic activation systems.

Role of metabolic activation in toxicology
In vitro studies. 2.

- Co-incubation of splenocytes with liver microsomes
  - Ethyl carbamate
  - Dimethylnitrosamine(DMN)
- Splenocytes: target cells
- Liver microsomes: metabolic activation system

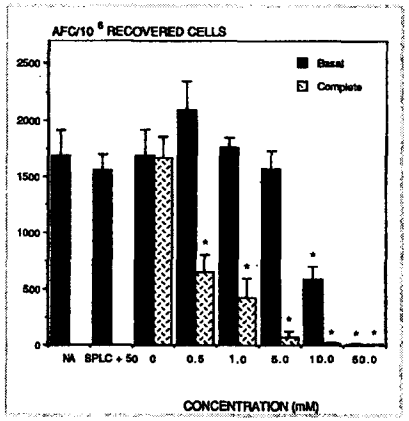
Role of metabolic activation in toxicology
In vitro studies. 3.

- Major disadvantages:
  - No phase II systems in liver microsomes
  - Microsomes are immunosuppressive when the incubation is prolonged.
  - Certain immunotoxicants could not be detected.
    - Ex., dimethylnitrosamine

Role of metabolic activation in toxicology
In vitro studies. 4.

- Hepatocytes/splenocytes coculture system to detect immunotoxicants which requires metabolic activation
  - Hepatocytes: anchorage-dependent cells act as a metabolic activator
  - Splenocytes: anchorage-independent cells act as a target cells
  - Splenocytes can easily be separated from hepatocytes after the coculture

Suppression of antibody response by dimethylnitrosamine in a coculture of murine hepatocytes and splenocytes



Future studies

- To develop immunotoxicity testing panels using human immune cells in vitro
- To develop human hepatocytes/human lymphocyte culture systems for detecting immunotoxicants requiring metabolic activation
- To develop methods for detecting toxicant-induced formation of DNA adduct in human lymphocytes

Conclusion

- Antibody response & flow cytometry may be able to be developed as biomarkers to assess chemical-induced immunotoxicity in human.
- In vitro metabolic activation system may be applied for detecting immunotoxicants which require metabolic activation for their toxicity.