

The Micromass Test - Method of Brown INVITTOX n° 122

Developmental toxicity

This method uses rat micromass cultures of limb bud and detects the inhibition of cell differentiation and growth, which are parameters suitable for teratogenicity testing.

Objective and Applications

TYPE OF TESTING	: Screening
LEVEL OF ASSESSMENT	: Toxic potential, toxic potency; Hazard
PURPOSE OF TESTING	: Classification and labelling, ranking

The Standard Operating Procedure presented in this protocol was evaluated in the ECVAM embryotoxicity validation study (1996-2000). The positive outcome of the study confirmed the possibility for the use of the test within the context of OECD test guideline 414, and Annex V, part B of the EU-Dangerous Substance Directive, for reducing and/or refining the use of animal procedures (Anon., 1988 and 2002).

According to the study outcome, the Management Team concluded that, rather than representing a complete replacement, the test should be used in the context of testing strategies for identifying strongly embryotoxic chemicals (Genschow *et al.*, 2002).

Several SOPs for the micromass assay exist and there was no optimal design of the test in the past.

The SOP presented in this INVITTOX protocol n.122 is most like that presented in INVITTOX protocol n.114. However, this SOP is designed with a maximum data per animal as the primary concern, followed by simplicity, scope for automation and economy as secondary criteria. In addition, a number of decisions were taken *a priori* referring to the choice of species, cell type and activating systems.

Rationale

Micromass is based on the technique devised by Umansky (1966) to study the development and differentiation of cultured chick embryo limb cells.

It was shown that the undifferentiated mesenchyme cells of limb buds will form foci of differentiating chondrocytes in micromass culture. Flint (1983) later introduced the use of central nervous system (CNS) cell culture.

The present method is based on detecting the ability of a particular chemical to inhibit the formation of foci. Thus, positive chemicals will reduce the number of foci, or the number of cells within foci.

The primary culture of limb bud cells of mammalian origin reproduces cartilage histogenesis, a fundamental step in the morphogenesis of the skeleton. Various functions, including cell proliferation, cell differentiation, cell to cell communication and cell to extracellular matrix interactions are implicated in this developmental process. Interference with these basic cell developmental functions may provide primordial teratogenic endpoints, and so this simple cell culture system appears to be a good model with which to study the teratogenic potential of chemical compounds.

Experimental Description

Endpoint and Endpoint Measurement:

CELL DIFFERENTIATION : Alcian blue staining (cartilage-specific proteoglycan stain)
CELL VIABILITY
CELL NUMBER
NEUTRAL RED UPTAKE

Endpoint Value:

ID50: 50% inhibition of cells differentiation and number of foci;

IC50: 50% inhibition of cell viability and growth

Experimental System(s):

LIMB BUD CELLS (rat embryo): Undifferentiated rat embryo limb bud cells

Basic procedure

Embryos are obtained from Wistar rats on day 14 of gestation and the limb buds are isolated. Single cell suspension is prepared by trypsin action. The subsequent step, spotting of cells into 96 well plates, is the most critical: place with care the spot in the centre of the well and make the volume and number of cells within the spot as consistent as possible. Place the 96 well plates in the incubator, then add medium with or without test chemical and leave them for 5 days. At the end, the total number of viable cells (i.e. IC50: 50% inhibition of cell viability and growth) and of differentiated cells (i.e. ID50 : 50% inhibition of cells differentiation) and number of foci were determined.

In devising this present protocol for the ECVAM Validation Study the following decisions were taken, a priori.

- rat embryos are used, to concur with orthodox regulatory testing and with parallel whole embryo culture studies. There does not appear to be any sound biological reason for choice of species, and there are arguments in favour of the chick (Brown et al. 1992).
- limb bud, only, is used as a source of cells.
(An analysis of previous data showed that using a second tissue detects a small number of additional chemicals. However, prevalidation studies showed neural cultures to be too variable).
- metabolic activation is not used. (An analysis of previous data shows that the addition of an activating system detects few additional chemicals).

In addition, a systematic series of experiments evaluated the following variables and their inter-relationships: size of culture vessel (12, 24, 48 or 96 well plates); spot size (5 to 20 μ l); cell density (0.25 to 2 x 10⁷/ml); chemical exposure period; serum concentration (5 or 10% FCS); media volume; media changes; antibiotics; staining methods.

Data Analysis/Prediction Model

In the micromass test two endpoint values are determined: ID50 for cells differentiation and IC50 for cells viability/growth. However, it was reported that because biostatistical evaluation proved that both of them provide the same information, in the Prediction Model developed in the preliminary phase of the ECVAM formal validation study only ID50 was used.

The model classifies the test chemicals into three classes of *in vitro* embryotoxicity: *non-*, *weakly* and *strongly*.

To compare the *in vitro* with their *in vivo* classifications, contingency statistics were obtained assessing the data accuracy, the method predictivity and precision for all three toxicity classes of the test chemicals.

(Anon., 2002; Genschow *et al.*, 2000 and 2002)

For further details see section 6 "Evaluation and Prediction Model" of the attached Procedure Details.

Test Compounds and Results Summary

Pharmaceuticals, agricultural and industrial chemicals, consumer products, food additives and contaminants (Brown, 2002).

Status

The SOP presented in this *INVITTOX* protocol has participated in an international (ECVAM) validation project: "***In vitro* tests for embryotoxicity: Prevalidation and validation of assays employing micromass cultures, rat embryo cultures and embryonic stem cells**" (1996-2000).

Based on the successful outcome of the study, the ECVAM Scientific Advisory Committee (ESAC) unanimously endorsed the statement that the results obtained in the definitive phase of the study with the Micromass test were highly reproducible, the correlation between *in vitro* and *in vivo* data was sufficient, and the test proved applicable for testing strongly embryotoxic chemicals.

The ESAC therefore agreed with the conclusion that the Micromass test is a scientifically validated test for identifying strongly embryotoxic chemicals and that it is ready to be considered for regulatory purposes.

(Anon., 2002).

An extensive search in the international scientific literature has been carried out, but no new articles referring to the use of the specific *INVITTOX* protocol No. 114 have been found since 2002.

Last update: April 2006

PROCEDURE DETAILS, 13 January 1999*

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Note: the protocol presents the standard operating procedure used in the validation study: "In Vitro tests for embryotoxicity: prevalidation and validation of assays employing micromass cultures, rat embryo cultures and embryonic stem cells".

* This SOP has been sent to the person responsible for the method to update or confirm the content. As soon as new information will be available this version will be updated.

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1. Materials

1.1. EQUIPMENT

- Tissue culture incubator, 37°C, humidified, 5% CO₂ in air.
- Horizontal laminar flow hooded bench (or other sterile environment)
- Heating block, 37°C
- Water bath 37°C
- Binocular dissecting microscope
- Inverted phase contrast microscope, with low power objective
- 96 well plate spectrophotometer, with 540 and 620nm filters (e.g. Titertek Multiscan)
- Haemocytometer (e.g. orthodox improved Neubauer type)
- 8 channel repetitive pipettor (e.g. Sealpette 50-1200. Fisher No. PMP-410-09ON)
- Single-channel repetitive pipettor (e.g. Eppendorf Multipette plus. Merck No. 30718550100)
- Iridectomy scissors with 45° blades
- watchmaker's No. 5 forceps

plus orthodox tissue culture, biochemical and minor surgical equipment.

1.2. STERILE PLASTICWARE

- 10 µm nylon mesh
- Swynex 1cm filter holders
- 96 well flat bottom tissue culture treated plates (Falcon No. 3072)
- 6ml round bottom tubes with cap (Falcon No. 2058)
- 60x15mm petri dish, non tissue culture (Sterilin)
- plastic transfer pipettes plus orthodox pipette tips, eppendorf tubes, syringes

1.3. NON-TEST CHEMICALS

1.3.1. Commercial chemicals and solutions

- Hanks' balanced salt solution, with phenol red (HBSS, Sigma No. H 6136)
- Ham's F-12 nutrient mixture (Sigma No. N 6760)
- Fetal calf serum (ICN/Flow No. 2910149)
- Penicillin G/streptomycin 100x liquid in saline (e.g. Gibco/BRL/Life Technologies 15140-031) Trypsin 10x liquid (e.g. Gibco/BRL/Life Technologies 25090-010)
- Neutral red (e.g. Sigma No. N 4638)
- alcian blue (Sigma No A 3157)
- 37% Formaldehyde (e.g. Sigma No. F-1268)

1.3.2. Storable solutions - sterile

- Ca/Mg free phosphate buffered saline (CMF). Store at 4°C, up to 2 months.
- 1.0% trypsin in CMF. Store at -20°C, up to one year.

1.3.3. Storable solutions - non sterile

- 0.9%w/v Saline. Store at room temperature.
- 0.4 % neutral red in sterile distilled water Store at 4°C, up to two weeks.
- 1% w/v alcian blue in 0.1 N HCl. Store at room temperature, up to two months. Filter through Whatman No.1 paper immediately before use
- acid alcohol: 1.0%v/v acetic acid in 50% ethanol. Store at 4°C, up to 2 months.
- Formol-Calcium: 1ml 37% formaldehyde plus 10ml of 0.1g/ml calcium chloride dihydrate, made up to 100ml with distilled water. Store at 4°C, up to 2 months.

1.3.4. Non storable solutions

Neutral Red: dilute 0.4% NR in medium, then incubate at 37° C overnight, or over weekend. If there is any sign of suspension or ppt, centrifuge at 2,000g for 10 to 20 minutes, then 0.22µm filter. If still any sign of suspension or ppt, repeat centrifugation and filtration. Use immediately.

6M Guanidine hydrochloride in water. Use immediately.

1.3.5. Positive and negative control test chemicals

Penicillin G (benzylpenicillin), Sodium (Sigma PEN-NA)

5-fluorouracil (Sigma F-6627)

1.4. CULTURE MEDIA

Hanks' BSS and Ham's F-12 are prepared according to the supplier's instructions.

Make medium as: 5%v/v FCS in Ham's F-12, add 10ml 100x pen/strep per litre then 0.22µm filter sterilise. Store at 4°C, up to 1 month. Although perhaps not ideal, we have decided to add penicillin/streptomycin to the culture medium.

1.5. EMBRYOS FOR LIMB BUDS

The selection of rat strain is not critical. There is some evidence that the potency of chemicals may vary (Ward & Newall, 1990), but not overall performance of the test. We use mature Wistar rats, because the number of embryos/litter is large.

To maximise the information/animal, we are using both fore and

hind limb buds, and embryos towards the older end of the acceptable range.

The objective is to generate embryos of 45 ± 5 somites, equivalent to 13 days embryonic age (post-fertilisation). We recommend the lower half of this range (i.e. 40-45 somites) when possible, as these seem to stain more strongly with alcian blue.

Somites can be counted using the hind limb bud as a horizon (the somite opposite the rostro-caudal mid-point is number 28).

Embryos of the right stage, at a convenient time of the day, can be produced either by holding animals in a room with reverse lighting schedule, or by using a day-time mating period.

We house a male and female together at 09.00, checking for copulatory plugs at 12.00. We designate this as day 1 of pregnancy. We harvest embryos between 10.00 and 12.00 on day 14 of pregnancy.

2. Culture Method

2.1. ISOLATION OF LIMB BUDS AND MID-BRAINS

The time for killing the animals to plating the spot cultures should be kept to less than 3 hours. In this time, one experienced worker can conveniently handle 40-60 embryos, yielding up to 10 plates of spot cultures of limb bud cells.

Kill day 14 time-pregnant rats by CO₂ inhalation followed by cervical dislocation (or other method approved by local regulations). Lay supine, wet fur of abdomen with 70% EtOH, make two incisions from the mid-line at the level of the hind limbs to the edge of the rib cage on left and right sides. Reflect the flap of skin rostrally. With course forceps, grasp the uterus at its bifurcation, pull up, cut through the cervix, lift the uterus (trimming away fat) then free by cutting through close to each ovary.

Lay the uterus on paper towel, stretched out fully, with the mesometrial side on the paper. Holding the uterus at one end with fine forceps, slit through the whole length of the antimesometrial wall using fine scissors, being careful not to puncture the implantation sites, by keeping the tip of the scissors pointing slightly off-axis. The wall of the uterus will naturally peel away from the implantation sites, which can be freed by gently excavating through the junction with the uterus using curved forceps. Transfer the implantation sites to a plastic petri dish of HBSS. Take the dish to a laminar flow hooded bench, or other sterile environment.

Under a dissection microscope (with 10x eyepieces, and 6x or 12x objective) and using No 5 watchmaker's forceps remove the embryos from the implantation sites. This can be done rapidly by piercing the implantation site from side to side through the amniotic cavity, tearing open to reveal the embryo, then slicing through the vitelline and umbilical vessels.

Transfer the embryos, using a wide-mouth plastic transfer pipette, to a fresh petri dish of HBSS. Evaluate the embryonic stage, reject those outside the range 45 ± 5 somites, record the numbers rejected and retained.

Each limb bud is removed by a single cut, as close to the flank of the embryo as possible. We find this is best done by holding the embryo (using watchmaker's forceps) with the rump uppermost,

then placing the blades of scissors parallel to the flank. We use iridectomy scissors with 45° blades.

2.2. PREPARATION OF SINGLE CELL SUSPENSION

Transfer all the limb buds into fresh dishes of HBSS. Count and record the number of limb buds. Transfer the tissues into a 6ml plastic tube. Remove the remaining CMF, then add 1.0% trypsin in CMF (about 2 ml for 80 limb buds). Incubate for 20 min at 37°C. Stop the trypsin action by adding about 2 ml of medium (Ham's F12 with 5%FCS). At this point the tissue will be slightly sticky and stringy, so care must be taken during rinsing not to aspirate tissue. Remove the fluid, then rinse twice more with medium. Remove as much medium as possible, then add a measured volume of medium using a variable volume pipettor. Add 10µl medium/limb bud.

Using a 200 µl tip with fine aperture attached to a pipettor, repetitively pipette the tissue through the aperture, being careful to avoid drawing air into the fluid, so as not to create bubbles. Do this just enough times to disperse all visible pieces of tissue. Draw up all the suspension into a 1.2 or 5 ml syringe, record the volume, then pass through 10 µm nylon mesh into a clean 6ml tube. (We cut circles of nylon mesh to fit 1 cm Swynex filter housing, which can then be autoclaved). Using a clean 1ml syringe, measure the volume of suspension again, and record.

Take a 10 µl sample to a 6ml tube, dilute 20x with 190µl of Ham's F-12, mix, then count cells, for example in an orthodox haemocytometer, but any reliable method of cell counting is acceptable. By this protocol, in an average experiment, there will be in excess of 100 cells/large square of the haemocytometer, which is equivalent to more than 2×10^7 cells/ml suspension. Adjust the cell counts to 2×10^7 /ml for limb buds, by adding the appropriate volume of medium.

2.3. PLATING OF SPOT CULTURES

The time between mesh-filtering the single cell suspension and plating spots should be kept to a minimum, to prevent clumping of cells. The next step, spotting of cells into wells, is probably the most critical step in the whole procedure, and must be done with care to ensure reproducible results.

There are two objectives in spotting of cells into the wells of a 96-well plate; to place the spot in the centre of the well; and to make the volume and number cells within the spot as consistent as possible. To enable this, we use an Eppendorf Multipette Plus with 0.1 ml Combitips, which will deliver 18 x 5µl volumes (however, other strategies that achieve the two objectives are allowed). Before each filling of the tip with cell suspension in medium, make sure that the suspension is well agitated, so of even cell density. Expel the first 5µl back into the tube, then expel each subsequent 5µl to form a small droplet on the end of the tip, then gently touch the tip to the exact centre of the well to transfer the fluid. When each plate is complete, check wells for a centrally-located spot, and mark any that have spread or are displaced onto the side-wall. At the end of the experiment, these can be excluded from analysis.

Place the 96 well plates in the incubator for 2-3h, to allow the cells to attach to the plates. After 2-3h, add 300µl of medium, with or without test chemical, to each well, then return plates to

the incubator for 5 days. (We have found that differentiation is significantly enhanced by using 300µl of medium, compared to 200µl).

3. Treatment of cultures

3.1. SOLVENTS

For test chemicals, allowed solvents, in order of preference, are:

Culture medium

HBSS or PBS 1% v/v maximum

Dimethyl sulphoxide (DMSO) 0.5% v/v maximum, but preferably 0.125% or less

Ethanol 0.2%

All dilutions of a test chemical and the solvent control must contain the same concentration of solvent.

3.2. RANGE FINDING

Make a final concentration of 1000 µg/ml of the test chemical, or the maximum soluble concentration, using the recommended solvent and maximum concentration given on the test chemical bottle. Make seven 10-fold serial dilutions, e.g.: 1 mg/ml, 100µg/ml, 10µg/ml, 1µg/ml, 100ng/ml, 10ng/ml, 1ng/ml, 0.1ng/ml.

A single test at these concentrations is acceptable, provided all aspects of the experiment are in order. Otherwise, a second run is required.

3.3. RESPONSE FINDING

From the range-findings results, prepare 8 dilutions to cover the relevant concentration range.

To be an acceptable test, there must be at least 3 concentrations within the range of 90% to 10% of control differentiation (alcian blue) values, or alternatively, a minimum dilution factor of 1.5.

For each chemical, two completely independent experiments that meet the quality control requirements are required. These two do not necessarily have to use exactly the same concentrations. This independence includes the preparation of fresh test solutions. Stock test solutions may be prepared the day before an experiment starts.

A frozen stock of the negative control penicillin-G may be used. The positive control 5FU must be made fresh for each experiment.

3.4 POSITIVE AND NEGATIVE CONTROL

Each experiment must include one plate with the full range of positive control concentrations, and a column of negative controls.

Make stock solution of 500 µg/ml 5-fluorouracil in HAM's F-12. We find that 20-30 seconds in an ultrasonic bath aids dissolution. Dilute 1:500 in medium to 1000ng/ml, then serial dilute 1+1, 6 times, in medium, to give final test concentrations of 1000, 500, 250 & 125, 62.5, 31.25, 15.625 ng/ml.

Make stock solution of Penicillin-G sodium (Sigma No. PEN-NA) at 500µg/ml in medium.

3.5. PLATE LAYOUT

3.5.1. Test Chemicals

Because the outside wells of the plate may differ in their evaporation properties, these are filled with medium only, leaving 10 columns of 6 wells for cells. Left and right columns (2&11) should contain solventcontrols. Second column from left (3) should contain medium controls. The other 7 columns (4 to 10) should contain test chemical.

	1	2	3	4	5	6	7	8	9	10	11	12
A	b	b	b	b	b	b	b	b	b	b	b	b
B	b	S	M	T1	T2	T3	T4	T5	T6	T7	S	b
C	b	S	M	T1	T2	T3	T4	T5	T6	T7	S	b
D	b	S	M	T1	T2	T3	T4	T5	T6	T7	S	b
E	b	S	M	T1	T2	T3	T4	T5	T6	T7	S	b
F	b	S	M	T1	T2	T3	T4	T5	T6	T7	S	b
G	b	S	m	T1	T2	T3	T4	T5	T6	T7	S	b
H	b	b	b	b	b	b	b	b	b	b	b	b

3.5.2. POSITIVE AND NEGATIVE CONTROLS

One plate of positive and negative controls must be run in each experiment. Left and right columns (2&11) should contain medium controls. Second column from left (3) should contain the negative control, penicillin-G at 500µg/ml. The other 7 columns (4 to 10) should contain the dilutions of 5- fluorouracil.

	1	2	3	4	5	6	7	8	9	10	11	12
A	b	b	b	b	b	b	b	b	b	b	b	b
B	b	M	P	F1	F2	F3	F4	F5	F6	F7	M	b
C	b	M	P	F1	F2	F3	F4	F5	F6	F7	M	b
D	b	M	P	F1	F2	F3	F4	F5	F6	F7	M	b
E	b	M	P	F1	F2	F3	F4	F5	F6	F7	M	b
F	b	M	P	F1	F2	F3	F4	F5	F6	F7	M	b
G	b	M	P	F1	F2	F3	F4	F5	F6	F7	M	b

H	b	b	b	b	b	b	b	b	b	b	b	b
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4. Assessment at the end of culture

4.1. METHODS

For the following manipulations, an electronic multi-channel repetitive dispenser is very useful. We use a Sealpette 1200 model. In all the following steps where removal of fluid from wells is required, simple tipping-out followed by inversion of the tray and blotting onto thick paper towel is convenient. Alternatively, aspiration can be used. Where rinsing with saline is required, fill wells with saline from a wash-bottle, being careful to squirt saline onto the side-wall of the wells, not directly onto the cell spot. Alternatively, a multi-channel repetitive dispenser can be used.

4.2. MEASUREMENT OF TOTAL NUMBER OF VIABLE CELLS

- Remove the medium from wells
- Blot to remove as much medium as possible
- Add 200µl of 0.005% neutral red in medium at 37°C.
- Incubate at 2-3 hr at 37°C
- Remove neutral red
- Rinse three times with saline
- Add 200µl formol-calcium, wait for about 1 min.
- remove formol-calcium
- Add 200µl acid alcohol. Leave for 30-60 min on a mixing table, until all the neutral red is extracted from cells
- Read absorbance in Multiscan spectrophotometer at 540nm (550 is fine, 492nm is acceptable)

The eluted stain can be read directly in wells with the cells. We have shown that although the presence of cells increases the absorbance it does not alter the overall outcome.

4.3. MEASUREMENT OF NUMBER OF DIFFERENTIATED CELLS AND FOCI

- Remove acid alcohol/eluted neutral red
- Rinse three times with saline
- Add 200µl 1 % alcian blue in 0.1 N HCl

Leave overnight to stain

- Remove alcian blue
- Rinse three times with saline.
- The number of foci can be counted at this point
- Add 200µl 6M guanidine hydrochloride (freshly made)
- Leave for at least 2h to elute stain (make sure all blue stain is removed from cell foci leave longer if necessary. Overnight is acceptable.)
- Read absorbance in Multiscan spectrophotometer at 620nm

5. Quality Checks

5.1. CONTROL CULTURES

It is up to the leader of each laboratory to ensure that only data that meets all the acceptance criteria are submitted to the biostatistician, and

that all parts of each spreadsheet are completed. This included a concomitant acceptable 5-FU control plate.

For an individual plate, the difference between the mean solvent controls in column 2 and in column 11 must be less than 30%.

For an individual plate, the mean absorbance of eluted neutral red in untreated cultures should be within the range (to be confirmed).

5.2. NEGATIVE CONTROLS

The negative control of 500µg/ml penicillin should not differ from untreated cultures by more than 50%.

5.3. POSITIVE CONTROLS

For an individual experiment, the neutral red and alcian blue readings in cultures exposed to 1000-15ng/ml 5-FU should cover the full range 0-100% of controls.

6. Evaluation and Prediction Model

6.1. CALCULATION OF ENDPOINTS

Data from the 96 well plate reader should be input directly into the appropriate Excel spreadsheets, which will automatically calculate the appropriate values. Values for cells that were marked as not being spotted correctly should be deleted, leaving the cells empty. A comment should be entered in the remarks section of the spreadsheet indicating why cells were deleted. This is of importance for interlaboratory trials.

For further questions, please contact the author of the SOP, or the Management Team of the "In vitro tests for embryotoxicity: Prevalidation and validation of assays employing micromass cultures, rat embryo cultures and embryonic stem cells"; available from dbVas of ECVAM SIS.

6.2. PREDICTION MODEL

Concentration response curves were analysed and the two endpoints, cytotoxicity and inhibition of differentiation, determined. The *in vitro* test provided a total of four experimental variables which could contribute to distinguish between classes of embryotoxic chemicals.

Although no systematic difference was apparent between cytotoxicity and inhibition of differentiation, a stepwise selection of variables was performed using analysis of discriminance.

The log(ID50) was identified as an appropriate variable to be used in three linear discriminant functions:

Function I	$6.65 * \log (\text{ID}50) - 9.49$
Function II	$6.16 * \log (\text{ID}50) - 8.29$
Function III	$-1.31 * \log (\text{ID}50) - 1.42$

Discriminant analysis allows to determine algorithms to distinguish among the three classes of embryotoxicity *non*, *weak* and *strong embryotoxic*, according to *in vivo* data. (Genschow *et al.*, 2000).

Chemicals Classification

To precisely classify the chemicals according to the PM, the following procedure is applied: if the result of function I exceeds the results of function II and III, the chemical is classified *non embryotoxic*; if the result of function II exceeds the results of function I and III, the chemical is classified *weak embryotoxic*; finally, if the result of function III exceeds the results of functions I and II, the chemical is classified *strong embryotoxic*. (Genschow *et al.*, 2002 and 2000).

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