March 12, 2008/Day 1/In vitro comet 2

- Test chemicals used were same for the prevalidation
  - All chemicals were delivered by the VMT
  - Same lot of chemicals
- Preliminary experiment from Honma's laboratory (see 8.2)
- Honma's lab did not take duplicate culture (some simplified protocol)
  - TB method was useless direct after treatment
  - He explained the data (8.2) together with the MN data
  - MMC, no response at all, not reduced because the control value was too low to show the reduction

3

March 12, 2008/Day 1/In vitro comet 3

- S9 mix did not work properly in the comet assay even cylclophosphamide
  - Cytotoxicity was not enough strong
  - Neither MN induction
  - Kikkoman S9 worked/specification was almost equivalent between French (Biopredic) and Kikkoman
  - Merck worked
  - BR not worked in the first exptl and worked the following exptl
- TB cytotoxicity test was commonly used in the mammalian assay because everyone thought it was the easy way. Dr. Uno suggest that we need the selection of the cytotoxicity assay more carefully. We will discuss later after other laboratories presentation.

4

March 12, 2008/Day 1/In vitro comet 4

- BR presentation (8.4 short version)
  - Diffrences form the standard protocol
    - Normal agarose from bottom gel
    - Cell washed and re-suspended in chilled complete media electrophoresis at room temp (21 2C and 2-8C)
  - RCG (cell count and TB) and ATP for cytotoxicity: both showed similar and reliable response)
  - Neutral-diffusion assay was not successful: subjective and no information about cytotoxicity: no difference between patterns of B and C and suddenly NA (no cell) [triton X].
  - 23.67 vs 4.17 in ethanol between duplicate culture. Merck has also similar phenomenon. One explanation is scoring. Blinding, prefixed area to analyzed. Subjectivity. Density of cell in the agar might be important to get more consistent data. Sample preparation might be important: slide quality.
  - % tail showed big difference but tail migration???
  - Acceptance criteria is important and should be set.

5

March 12, 2008/Day 1/In vitro comet 5

- Merck presentation
  - No difference from the standard protocol
- Hedgehogs in control group showed 2-7%; ethanol 17, 17
- Some positive response were observed in MMC
- TB for cytotoxicity showed no sense response
- 2AA specific figure (hedgehog), core in the cloud
- Hedgehog can provide good information about cytotoxicity

6

March 12, 2008/Day 1/In vitro comet 6

- Huntingdon presentation
  - EMS showed good dose response positive but top two conc showed too many hedgehogs
  - Again, TB 0 day showed no sense for cytotoxicity. RCG was good indication.
  - MMC, at low conc increase comet but higher concs decrease comet dose-depending manner (control level was 14.22%): bell-shape curve
  - One example big difference between duplicate in % tail but not so big in tail length
  - 2-amino anthracene showed too cytotoxic in the presence of S9 mix (without S9 mix also showed less convincing outcomes; precipitation, hedgehogs). This chemical will be repeated.
  - Variation of control % tail (about 5 to 16%) cross studies

7

March 12, 2008/Day 1/In vitro comet 7

- Cell differences
  - TK6 cell has many advantages. E.g., human cell line, with normal P53 situation.
  - L5178Y cell has also advantage together with gene mutation assay
  - Human lymphocytes; variability among donors: low background
  - BR: although there are some differences we can use any cell types
- Why not include positive control in all experiments?
  - Because at the moment, we used clear positive and negative chemicals and no need positive control group.

8

March 12, 2008/Day 1/In vitro comet 8

- Hadano presentation
  - 2AA different conc was used. Other condition were followed the standard protocol
- Again TB immediately after treatment did not show any response
- S9, sometimes works but not always
- Cyclophosphamide needs higher conc to induce comet compare to the CA assay
- In vivo, comet and MN response are observed under the similar dose levels but not in vitro. In vitro, comet usually require higher dose compare to the MN assay.

9

March 12, 2008/Day 1/In vitro comet 9

- Anpyo presentation (only one chemical) EMS
- Again, TB 0 day did not work.

10

March 12, 2008/Day 1/In vitro comet 10

- Dr. Honma explain the standard data sheet to be used in the in vitro validation study
- 2 slide from 1 culture
- Dr. Suzuki is taking care of this.

11

March 12, 2008/Day 1/In vitro comet 11
To be discussed

- How to asses cytotoxicity
    - Endpoint/Treatment protocol
    - TB direct after treatment is no longer necessary.
    - TB 3-4 h after treatment can be used
    - RSG 24 h should be included
    - ATP assay has some limitations (optional use)
    - Neutral diffusion is not good indicator for cytotoxicity
- How to overcome inconsistency between duplicate cultures
    - Density of cells in agar
    - How to reduce subjectivity
        - Blinding
        - Automation without man decision to select cells to be analyzed
        - In vitro and in vivo issues
- Technical transfer, quality control of preparations
    - More detail protocol

12

March 12, 2008/Day 1/In vitro comet 12
To be discussed

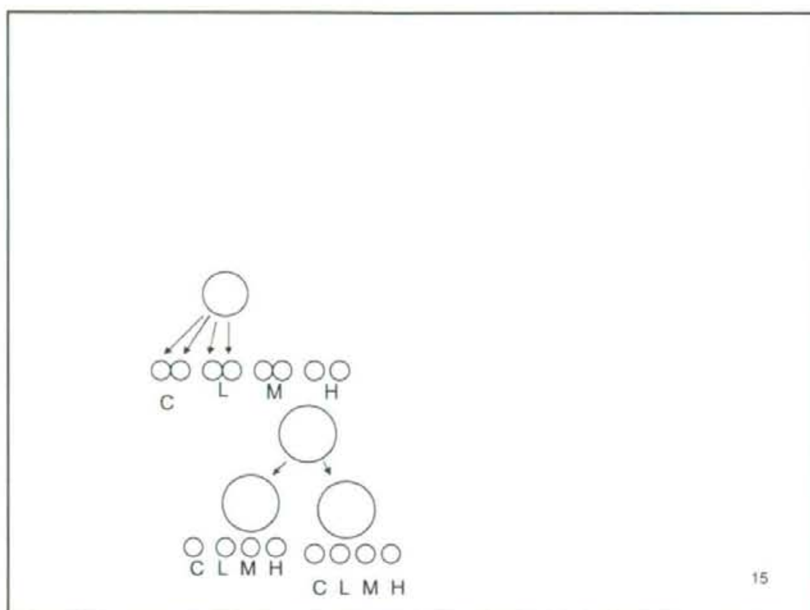
- To be discussed
  - Hedgehog
  - Special issue for in vitro assay. It is good indication of slide quality as well as cytotoxicity. Definition of hedgehog and comet: machine dependent. Another scan to count hedgehog among 100 cells.
- Cell type; TK6 is good enough or we have to think about the other cell types
  - Although TK6 is not perfect, we keep to use TK6 for the validation study
- Positive control
  - EMS as same as in vivo comet assay (250 μg/mL) in this validation study (expectation response: minimum 2 folds in % tail)
- S9
  - We have to try again using DMN and CYP (only with S9 mix).
    - 3-4 h treatment; MolTox S9 will be used
- Serum
  - FBS is recommended in the validation study.

13

March 12, 2008/Day 1/In vitro comet 13
To be discussed

- Positive definition; what we want to detect
  - Both in vitro and in vivo
- Duplicate culture
  - At least duplicate culture. To analyze the present control intra- and inter-laboratories.
- Electrophoresis; e.g., temperature
  - Both in vitro and in vivo
- How to score slides
  - Both in vitro and in vivo

14



March 13, 2008/Day 2/In vivo comet 1

- This section was leaded by Dr. Y. Uno
- Dr. Omori delivered graphs of all outcomes from the phase 2 validation. All participants, without statistician, marked + or – for each graphs. These intuitional judgments will be looked into by statistician group meeting after lunch.
- Dr. Omori asked participating laboratories to check again all data they provided.
- Dr. Omori showed the summarized data and explained the outcomes.
- Dr. Uno broke the code of chemicals, A, B, and C.
- Dr. Uno asked each laboratory to present data briefly.
- L-1 Merck, 2-BR, 3-HLS, 4-Hadano, 5-Anpyo

16

March 13, 2008/Day 2/In vivo comet 2

- Dr. Omori explained using figures. E.g., trend test, 5% both sides and pair-wise comparison (Dunnett test) 5% one side with (J_D) and without (J_L) consideration of multiplicity.
- We have to discuss whether one side or two sides test should be used for further evaluation. E.g., should we consider to detect the cross-linking agent.
- Fig. 7L
- Dr. Uno explained the terminology:
  - Endpoint: individual values of each parameter
  - Estimate: a mean calculated with values of endpoint
  - Effect: difference of an average of estimate between a control group and a treatment group.

17

March 13, 2008/Day 2/In vivo comet 3

- Fist, only EMS data were discussed in effect at each laboratory
- Lab 2 showed big variance comparing to other laboratories
- Average inter-laboratory was approximately 100
- Variability between laboratories was approx 300
- Intra-laboratory variation varied from 0 to 400

18

March 13, 2008/Day 2/In vivo comet 4

- Stomach showed much more variability compared to Liver
  - Average inter-laboratory was approximately 100
  - Variability between laboratories was approx 300
  - Intra-laboratory variation varied from 0 to 300
- Fig. 9 showed the differences between liver and stomach

19

March 13, 2008/Day 2/In vivo comet 5

- Merck presentation
  - #1 slight increase in liver and stomach at low dose
  - #2 slight increase in liver and negative in stomach
  - #3 negative in both liver and stomach
  - #3 showed slight change in liver at all dose but not in stomach in the high dose group (NE lower dose levels)
- The timing of treatment might be important especially for the stomach

20

March 13, 2008/Day 2/In vivo comet 6

- BR
  - Room temp > <10C
  - Peripheral blood was also analyzed
  - Acrylamide: Neutral diffusion showed slight effects in liver
  - Histopathology showed no effect in acrylamide, 2,4-dat some effects in liver
  - EMS exptl-2 high response, similar room temp and <10C in liver, stomach, and peripheral blood

21

March 13, 2008/Day 2/In vivo comet 7

- HLS
  - All chemicals showed dose-dependent increase in liver and stomach (2-3 fold increase)
  - These results were reproducible based on the repeat experiment
  - The positive increase were observed in BM, kidney (strong), and colon (only on comp 7 and 8)
  - Histopathology: acrylamide showed increase MI in liver, however no hedgehog
  - 2,6, showed no change
  - 2,4 increased apoptosis and single cell necrosis, and hypertrophy in liver
  - No effect in the neutral diffusion in liver and stomach

22

March 13, 2008/Day 2/In vivo comet 8

- Hatano presentation
  - Histogram showed pooled data
  - Acrylamide showed positive response in only the low dose in stomach but negative in liver
  - 2,4 and 2,6 showed negative
  - Histopathology showed no response on acrylamide/stomach, which was positive in comet
- Anpyo presentation
  - 2,6 negative
  - 2,4: deviation from the standard protocol that might be important
    - 1st treatment14:30 as same as standard but not EMS (no treatment) and next day 11:30 and 21 h later (8:30 on the next morning). Stomach was full at 8:30 and less effective for the treatment.
  - Acrylamide was positive in liver and stomach
  - No histopathological effect

23

March 13, 2008/Day 2/In vivo comet 9

- Treatment time at each laboratory
- BR: single treatment for EMS 3 h prior to kill animal
  - 9 and 6-7 am (not always fixed time; early morning)
- Merck: single
  - 9 am
- HLS: single
  - 8-9 am
- Hatano: double treatments
  - 14:30 and 11:30
- Anpyo: double treatments
  - 14:30 and 11:30

24

March 13, 2008/Day 2/In vivo comet 10

- Three groups, i.e., VMT, statistician, participants, had isolated meetings after lunch
- VMT discussed the further validation study strategy
  - After consultation with Ray and Len for the VMT proposal before showing to participants
- Laboratory sub-group (Dr. Brian)
  - Animal fasting: no
  - Electrophoresis: temp, voltage constant, etc.
  - Electrophoresis time: some variation; control tail length
  - Control % tail: consistent among animals, among experiments, among laboratories
  - pH: all >13
  - Vehicle: OK
  - Dose of positive control: OK

25

March 13, 2008/Day 2/In vivo comet 11

- Laboratory sub-group (Brian)
  - Animal fasting: no
  - Electrophoresis: temp,
  - Voltage: 0.7-1 volt/cm 22-36 cm box
  - mAmps: approx 300, constant among labs
  - Electrophoresis time: some variation; control tail length
  - Control % tail: consistent among animals, among experiments, among laboratories
  - pH: all >13
  - Vehicle: OK
  - Dose of positive control: OK
  - Tissue to cells: 3-60 min
  - Cells on slide: 3min to 2h
  - Lysing: overnight in dark cold
  - Estimation of cell number on slide: no count or estimate

26

March 13, 2008/Day 2/In vivo comet 12

- Laboratory sub-group (Brian)
  - Magnification and cell density: 200X 3-15 cells/field
  - How stable negative control data: quite many historical negative controls (send data to Dr. Omori for further analysis)
  - The range of positive control was so bad compare to the other assay?

27

March 13, 2008/Day 2/In vivo comet 13

- Statistician sub-group (Takashi)
  - Positive control: should be "clear" positive, for example:
    - Criteria EMS/VC >2, 3, or 5, or (VC: vehicle control)
    - EMS-VC >10, 15, or 20%
    - Lab provide negative control values
  - If protocol will be revised, this should be describe clearly
- Decision criteria (positive and negative judgment)
  - Should be made by VMT and laboratories
  - The criteria should be decided by the next study
  - Valiance of EMS is less important and the ratio/difference between EMS and VC is more important
- 24/30 called negative by toxicologist's intuition
  - 7/15 liver, called positive by trend test
  - 6/15 stomach positive

28

March 13, 2008/Day 2/In vivo comet 14

- Statistician sub-group (Dr. Omori)
  - Further validation study
  - To examine whether the finalized protocol will work or not, conducting the study by these 5 lab. Is one of ways before a large laboratory study.
  - All 5 leading laboratories necessary for all compounds? One chemical per 3 laboratories?

29

March 13, 2008/Day 2/In vivo comet 15
To be discussed

- Cytotoxicity: neutral diffusion, histopathology
- Number of slide/animal (2-3)
- Number of animals/group (power calculation: criteria of positive and negative)
- How to analyze slides (both for in vitro and in vivo)
- Publication of this validation study (Uno-san will draft the paper)

30

March 14, 2008/Day 3/Summary 1

- This section was leaded by Dr. Y. Uno
- Dr. Uno explained the outcomes from the VMT meeting briefly including the future plan and phase 3 and 4 validation activities.
- Consensus: We will use only % DNA in tail. Neither the tail length nor Olive tail moment give additional information to the overall evaluation comet assay
- Dr. Uno also explained the acceptance criteria that proposed from VMT and also statistician
  - Acceptable valiance within laboratory is <100 and <15% DNA of tail in the negative control (VMT)
  - Positive control shows 'clear' positive: 2, 3, or 5 fold increase to the negative control/Effect (EMS-negative control) > 10, 15, 20% of DNA of tail

31

March 14, 2008/Day 3/Summary 2

  - Dr. Lovel claimed the suggestion from the VMT is not understandable. Needs practical decision should be considered
  - The suggestion from VMT is too strict and it will be happened several times to reject experiments
  - Dr. Hayashi emphasize the quality control of the comet assay by the acceptable level of negative control should be less than 15 % DNA in tail and for the valiance within laboratory of positive control (EMS 200 mg/kg) should be 100 (3 sigma)
  - The advice by statistician should take into consideration
  - There are some misunderstanding of VMT proposal to the statisticians. The CV may be better parameter for this purpose.
- The matter should be discussed again among all members (including Ray and Len) of VMT to finalize

32

March 14, 2008/Day 3/Summary 3

- Practical plan for the in vivo comet validation study
  - The name of the validation study
    - "1st phase validation study" instead of "1st pre-validation study"
    - "2nd phase validation study" instead of "2nd pre-validation study"
    - "3rd phase validation study" instead of "Main validation study"
- Check all data by the laboratory and report to Dr. Omori
- Laboratory precise protocol and final report submit to Dr. Uno

- Minor revision on the standard protocol, ver. 12
  - Supplier of low-melting agarose
  - Tissue sampling time: 3 h after 2nd administration
  - Slide dehydration: possible to skip this process if sides are scored very soon

33

March 14, 2008/Day 3/Summary 4

- Practical issues
  - EDTA addition is not required into low-melting agarose gel in the validation study
  - Sensitivity is higher running at room temperature. We will wait for the outcomes experiments will be done by Andrew.
  - [Tentative agreement: Electrophoresis solution temp: <10C (or range should be set, e.g., 5-10C) (electrophoresis buffer)
    - Do not put ice cubes into the buffer]
- % DNA in tail in negative control:
  - It depend on the tissues: e.g., liver, lower is better and usually less than approximately X % but stomach less that approximately Y%. Statistician can say the values by the analysis of all control data from the 2nd validation study. Historical control data will be helpful.

34

March 14, 2008/Day 3/Summary 5

- How to analyze slides? Which area of slide should be analyze? Optimal cell density? The issue was discussed among all members including statisticians.
  - There is not so much influence to the test result. The important is to avoid overlap scoring, outside area, <5 cells/area should be analyzed, at least 10 area.
  - The cell density might be important to be controlled especially in vitro assay but not so important in vivo study.

35

March 14, 2008/Day 3/Summary 6

- Measure cytotoxicity
  - Histopathological examination will be included. How to use histopathological data for interpretation of comet assay will continue to discuss. Negative or positive in comet? Histopathological data will need only when the comet assay is positive.
  - Neutral diffusion no more necessary in the comet assay.
- Electrophoresis
  - Is it necessary to set the electrophoresis condition (e.g., voltage and time) should be fixed more strictly?
  - Brian will send the data to Dr. Uno showing big difference between 0.7 *vs* 1.0/cm.
  - Brian proposed to use consistent condition (e.g., 0.7 and 20 min) in the next trial (after analysis of the data provided by Brian).

36

Draft Minutes

The 5th meeting for

the International Comet assay validation study

Date: February 4, 2009, 9:00 a.m.-February 6, 3:00 p.m.

Venue: Sango and Suisho, 3F, Kansai Airport Washington Hotel, Osaka, Japan

February 4

Welcome address (Chair: Dr. Makoto Hayashi: Anpyo-Center)

1. The chair opened the meeting and welcomed participants. He introduced the topic and gave a presentation on the final goal of this project and the object of the 5th meeting for the International Comet assay validation study in Osaka, 2009 (Osaka meeting). He described the final goal of this project establishes the OECD Test Guidelines of comet assay and the object of the Osaka meeting is to understand the current situation of *in vitro* and *in vivo* comet assay.
2. The chair emphasized that participants ideally are present as members of the Validation Management Team (VMT) or the delegate of the participated laboratories. The Chair introduced Dr. Masamitsu Homma (NIHS) and Dr. Yoshifumi Uno (Mitsubishi Tanabe Pharma Corporation) as the acting co-chairs together with the chair, who were approved by the meeting.
3. The chair also announced that the standard protocol ver. 13 should be revised in accordance to the activity of ICH, EFPIA initiative collaborative study of comet assay incorporated into repeat-dose general toxicity study to fulfill the 3Rs of animal experiment. Namely, 3 treatments (0, 24, and 45 h) before sacrifice animals at 48 h after the first treatment instead of 2 treatments describes in the protocol ver. 13. This protocol can be used for the combination study of the micronucleus assay and the comet assay.
4. Participants introduced themselves to the meeting (for a list of participants see Annex 2).
5. Dr. Hajime Kojima (JaCVAM, NIHS) explained the general information in this meeting.

In *vitro* Comet-international validation study (Chair: Dr. Honma)

To understand the current situation of *in vitro* comet assay

1. To summarize Phase I

The mainly phase I data were presented by Dr. Honma.

Statistical consideration on phase I study by Mr. Masaya Suzuki (Anpyo-Center)

- 5 laboratories and 5 chemicals/laboratory were presented.
- 2AA: Variation may depend on different S9mix (only 2 +ve)
  - Hatano Research Institute (HRI) showed data that the different S9mix showed different results
- For the statistical analysis, one tail vs. two tails was discussed: no concrete conclusion.
- Flow: wording mean of relative cell survival ...
- Log transformation for concentration was proposed by Dr. Lovell to show clearer dose-response relationship: Action, re-calculation and re-draw graphs by M. Suzuki.
- Failure based on protocol ???
- Generally, the variation in the comet assay can be smaller than Chromosome aberration assay.

2. To discuss on Phase II

The phase II study has not yet completed and not be able to break codes of chemicals. Thus, we could not discuss precisely based on the chemical information.

- 3 laboratories completed (dead line by the end of March)
- Presentation by the participating laboratories
- BR (BioReliance Corporation) had problem of KCl which for without S9 mix, then they used culture medium instead of KCl (They want to repeat experiments without S9 mix)
- Acceptance criteria: Hedgehog, >20%, the all concentration above that showed >20%
- Negative control. Solvent, KCl/S9 mix

By the end of May, all data should be collected

3. To discuss the future activities

Discussion and consensus

For Phase III studies, number of laboratories and number of chemicals were discussed. The plan will be proposed by the VMT.

February 4

Workshop - Image Analysis with Comet IV (Chair: Dr. Hayashi)

Dr. Hayashi expressed the importance of distinguishing between hedgehogs that is not genotoxic but related to cell-death, e.g., apoptosis and necrosis, and genotoxic comet strong positive cells. This may lead false-positive, which is the biggest concern of this assay. The group basically agreed the importance of the identification of the non-genotoxic hedgehog and understood this workshop. The main purpose of the workshop is to make consensus of the definition of hedgehog

- Comet and Hedgehog, which is which? : Dr. Madoka Nakajima and Dr. Jin Tanaka (Anpyo-Center) prepared the example images and all participants voted their judgment by their experience and intuition.
- After the vote to about 100 images, all questionable images were discussed one by one and discussed and agreed. All agreed the importance of definition of hedgehog but clear definition could not be made. The practical consensus at the moment for the phase IV validation, all images that image-analyzer captures and analyses will accept as genotoxic comet positive nuclei.
- VMT and Consultation team will prepare a color atlas for reference to distinguish between Comet and Hedgehog as soon as possible to send all participants for the phase IV before their starting analyses.

February 4

VMT meeting

Discussion and consensus (Chair: Dr. Hayashi)

1. In vitro comet assay

- Cytotoxicity: Caspase 3,7; ATP, 4 and 24 h
- Data acceptance criteria (duplicate)
- Assay acceptance criteria
- Identical to the *in vivo* criteria
- Negative control range, etc. (historical control +/- 2SD)
- Positive control response (EMS, one conc. In addition a chemical which need metabolic activation, DMN)
- KCl issue (BR will provide new data by the end of May)
- Evaluation criteria
- How many chemicals for phase III?
- Identical chemicals to *in vivo* assay.

2. *In vivo* comet assay

- ✓ Four groups have their common chemical (phase IV-a) + each laboratory will have 3 chemicals uniquely (phase IV-b).
- ✓ Each group includes a leading laboratory.
- ✓ Four common chemicals will be done first (phase IV-a) and labs provide data as soon as possible. We may make adjustment protocol and others, if necessary.

VMT should consider a possibility that a few labs may withdraw from this validation effort.

- ✓ **February 5**

In vivo data analysis and next study plan (Chair: Dr. Uno)

1. Introduction - Dr. Uno summarized the outcome of phase II

- Note: to correct 'Dunnett_2' described in the handouts to 'Unrestricted least significant different method'
- There was some inconsistency of results among laboratories in the phase II study. Therefore, the phase III study was conducted using the revised protocol v.13.

Discussion about data acceptance criteria:

- ✓ Negative Control

  Liver 1-8%: reasonable based on the mean +/- 3SD

  Stomach 1-30%: too wide, and 6-20% seems reasonable based on the mean+/- 3SD in 4 labs

- ✓ Mean value should be in the range
- ✓ Positive control should be statistically positive [not to indicate the actual values]
- ✓ SD should be better than CV
- ✓ EMS 200mg/kg
- ✓ 2-fold or higher, 5 or higher and CV less than 50% in both liver and stomach

2. Data analysis of the phase III validation study

Dr. Uno summarized the phase III study

- Protocol version 13 was applied
- Chemicals: MNU (Chemical D), EMS (Chemical E), D-mannitol (Chemical F) + Positive control: EMS

Dr. Takashi Omori (Kyoto Univ.) explained the statistical analysis results

- Graphs in handouts reveal the mean value and the confidence interval instead of the S.E.
- All negative control groups showed the averages within the draft data acceptance criteria.
- All positive control groups were increased in the effects (difference and ratio) with statistical significance (t-test, $P<0.05$) which met the draft data acceptance criteria except for the effect (diff.) in the liver at Lab.4.
- MNU and EMS showed statistically significant results with the Dunnett test (both side, $P<0.05$) and the trend test ($P<0.05$) in all laboratories. D-Mannitol showed no statistical significance in all laboratories.

Discussion

- The reasons why magnitudes of the effects were different among labs? ==> Probably related to electrophoresis duration: Lab.1 20 min, Lab.2 30 min, Lab.3 20 min in liver & 30 min in stomach, Lab.4 15 min.

Comment to the acceptance criteria (Dr. Tice)

Keep the electrophoresis condition including 0.7V, 300mA, and duration (20 min) constant, positive control should be statistically positive (effect-difference). Negative control should be evaluated based on the laboratory's historical control database (at the beginning set guide number, e.g., 1-8% for liver and 3-20% for stomach). If the data cannot meet the above tentative criteria, then the adjustment of the electrophoresis condition will be needed.

Positive control

Revise from one administration to two or three administration (24, 45h, or 0, 24, 45h) and sampling at 3h after the last administration using 3 animals instead of 5.

Set global cut off value for the positive response based on the analysis of the distribution of negative controls, e.g., mean + 2SD.

3. The selection of new laboratories to participate in the phase IV collaborative study.

Dr. Uno summarized the selection of new laboratories to participate in the phase IV. The 13 laboratories will join this validation effort including the leading laboratories.

4. Plan of the phase IV

- Purpose

Predictivity

Integration and/or combination

- Standard protocol version 14 (now preparing)

Three times administration at 0, 24, and 45 h and kill animals 3 h after the last administration. Positive control (EMS) will be also administered two times as same as test articles. (24 and 45 h and kill animals 3 h after the last administration (micronucleus (MN) in bone marrow will be detected in two times administration of EMS. If peripheral blood will be used, the 3 administrations (0, 24, and 45h) will be necessary).

The MN and Comet analysis in bone marrow/peripheral blood is just optional in this validation study.

The solid description about the condition of electrophoresis should be included in the protocol ver. 14.

Parameter: only % tail DNA but not tail length (should be defined, if used: tail migration or tail length by Comet IV) either Olive tail moment will be used. All data should be kept at each laboratory and only % tail DNA data should be submitted (keep the data of other parameter should be kept in the database for further use, if necessary).

- Delivery of chemicals is VMT matter (regional distribution may be considered).
- Common controls (one negative cont. + one positive cont.) are acceptable when two test chemicals are examined in one experiment.
- End of the validation study will be by the end of December, 2010.
- A workshop: image analysis, *e.g.*, comet *vs.* hedgehog.

  Now planning to hold a one (or two)-day workshop sometime in conjunction with the 10th ICEM 2009 - INTERNATIONAL CONFERENCE ON ENVIROMENTAL MUTAGENS, 8th International comet assay workshop or 7th World Congress on alternatives and animal use in the life sciences in August-September. At the workshop, technical questions by the participants will be also discussed. Data of the first experiment may be discussed if the data are available by the end of June (==>VMT matter? If chemical delivery by March then possible? Probably difficult due to both reasons of chemical delivery and lab acceptability)
- Some technical Q&A
- Two slides/tissue vs. three slides—>Two (Note: Data from Merck (two vs. three slides) should be analyzed)