Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Res. Virol.
1992, 143, 231-240

The pattern of proteins synthesized in the liver is profoundly modified upon infection of susceptible mice with mouse hepatitis virus 3

M.A. Lucchiari [1, 2], C.A. Pereira [1], L. Kuhn [3] and I. Lefkovits [3] [*]

[1] *Instituto Butantan, Laboratorio de Imunologia Viral, Sao Paulo (Brazil),*
[2] *Max-Planck-Institut für Immunbiologie, Freiburg (Germany), and*
[3] *Basel Institute for Immunology, Basel (Switzerland)*

SUMMARY

Susceptible BALB/c mice, after experimental infection with mouse hepatitis virus 3 (MHV3), revealed virus titres in the liver that increased gradually to a peak of 8×10^5 PFU/g of tissue after 3 days' infection, when the mice died of acute hepatitis. BALB/c mice were infected with MHV3, subsequently labelled *in vivo* with ^{35}S-methionine, and then the liver preparations from both infected and non-infected animals were subjected to two-dimensional gel electrophoresis. Comparisons of the patterns by computer image analysis revealed 17 gene products which increased, and 8 gene products which decreased, upon virus infection in their two-dimensional gel spot intensity. We conclude that during MHV3 infection of a susceptible strain of mice, a major modification in protein synthesis occurs. The pattern alterations were not related to the virus gene products but were mostly endogenous mouse proteins. Whether these proteins are a result of a defence attempt by the animal, or are dictated by the virus in order to prevent a protective response from happening, remains to be shown.

Key-words: Coronavirus, Mouse hepatitis, Protein; Synthesis, Liver, Virus 3, *In vivo* labelling, Defences.

INTRODUCTION

Coronaviruses cause a wide variety of diseases in several hosts (McIntosh, 1985). The murine hepatitis virus (MHV) group is widespread, occurring endemically in mouse colonies. MHV3 was isolated by Dick *et al.* (1956) and has been used as a model of viral infection in which resistance is dependent on the genetic background of the mouse strain (Arnheiter *et al.*, 1982; Dupuy *et al.*, 1984; Levy *et al.*, 1981; Lucchiari and Pereira, 1989, 1990; Virelizier and Gresser, 1978). Adult BALB/c mice are susceptible to MHV3 infection. After intraperitoneal (i.p.) inoculation with the virus, the animals develop acute hepatitis and die 3 to 5 days later. In the peritoneum, the virus replicates in the macrophages and reaches the liver via the blood stream where it replicates first in the Kupffer and endothelial cells of the liver sinusoid, and then

Submitted March 25, 1992, accepted July 10, 1992.

(*) Corresponding author.

continues to replicate in the hepatocytes (Arnheiter *et al.*, 1982; Bang and Warwick, 1960; Pereira *et al.*, 1984a).

A large number of local and systemic changes have been shown to be involved in the resistance of animals following infection (Eaton *et al.*, 1982; Weinberg, 1984). The changes in the concentration of plasma proteins are an important factor of the systemic response (Kushner *et al.*, 1982). Synthesis or degradation of several plasma proteins are affected during infection and the change in components of the acute phase reactants is a prominent feature of the response. "Positive" and "negative reactants" are terms that have been proposed to describe the increase or decrease in the abundance of individual components during the acute phase (Kushner, 1981). A wide variety of different stimuli can trigger an acute-phase reaction, and some of the inducers evoke a distinct response pattern (Allen *et al.*, 1982). A number of other changes in protein synthesis have been implicated, but have not been revealed until now, since no direct, sensitive methods were available for the detection of polypeptides present at concentrations considerably lower than those of acute-phase proteins.

Two-dimensional gel electrophoresis, which allows simultaneous qualitative and quantitative analysis of numerous proteins in combination with *in vivo* labelling, turned out to be an ideal tool for the detection of changes (Pluschke *et al.*, 1986) directly related to a course of infection.

The results presented here show the changes observed in liver and serum proteins of BALB/c mice during infection with MHV3.

MATERIALS AND METHODS

Virus

MHV3, originally obtained from Dr. J.L. Virelizier, Institut Pasteur, France, was cloned by limiting dilution. One plaque was selected and amplified on L929 cells to serve as the inoculum for future stocks (Martin *et al.*, 1988) to limit spontaneous mutations. The stocks were titrated by plaque assay on L929 cells, as previously described (Pereira *et al.*, 1984b). Aliquots containing 2×10^5 plaque-forming units per millilitre (PFU/ml) were stored at −80°C for use in the experiments.

Mice

BALB/c mice (4 to 8 weeks old) from the animal colony of the Max-Planck-Institut, Freiburg, Germany were used in all the experiments.

Experimental virus infection

MHV3 (10^3 PFU) was inoculated intraperitoneally. The mice were observed during the following 3 weeks and the mortality was recorded. In order to evaluate the virus growth kinetics in the liver during infection, selected mice were killed periodically, their livers removed, ground and resuspended in 2 ml of RPMI-1640 medium containing 5 % of foetal calf serum (FCS) and the virus titres determined by plaque assay on L929 cells (Pereira *et al.*, 1984b). The *in vivo* experiments were performed at the Max-Planck Institut für Immunbiologie, Freiburg, Germany.

Labelling procedure

The labelling procedure has been described in detail elsewhere (Pluschke and Lefkovits, 1984). Briefly, the animals were inoculated intravenously (i.v.) with 10^3 PFU of MHV3, two days later they were injected i.v. with 1 mCi of ^{35}S-methionine (Amersham International, UK), with a specific activity of 1,000-1,500 KCi/mol, in 100 µl of isotonic saline for a period of 4 h. The mice were killed and the liver and serum removed.

Sample preparation

Liver slices (3-10 mg) or serum samples (10 µl) were placed in Sarstedt tubes and instantaneously solubilized by mixing with 90 µl buffer containing 20 g/l of NP40 detergent (BDH, Zürich, Switzerland), 10 g/l 2-mercaptoethanol (LKB, Bromma,

2D-SDS-PAGE = two-dimensional sodium dodecyl sulphate/polyacrylamide gel electrophoresis.
FCS = foetal calf serum.
IEF = isolelectric focusing.
i.p. = intraperitoneal(ly).
i.v. = intravenous(ly).
MHV3 = mouse hepatitis virus 3.
PFU = plaque-forming unit.

Sweden) in 9 M urea (Schwartz-Mann, Orangeburg, NY, USA) and adjusted to pH 9.5.

Two-dimensional gel electrophoresis (2D-SDS-PAGE)

The labelled materials were subjected to 2D-SDS-PAGE by a previously described procedure (Lefkovits *et al.*, 1985) based on the system originally developed by O'Farrell, 1975. In the first dimension, isolelectric focusing (IEF) from pH 3 to 9 was used. In the second dimension, SDS-PAGE with a 10 to 20 % polyacrylamide gradient was used. Both dimensions were run under reducing conditions on an "ISODALT" apparatus, which is described in detail elsewhere (Tollaksen *et al.*, 1981), as is the composition of the gel components and their preparation (Lefkovits *et al.*, 1985).

Radiofluorography

Gels were immersed twice for 1 h each time in dimethyl sulphoxide (Merck), then incubated at room temperature for 3 h in a solution of 2,5-diphenyloxazole (130 g) dissolved in dimethyl sulphoxide (1 l). The gels were dried (3 h, 50°C, reduced pressure) and placed in contact with "XAR-5 Kodak" film at −70°C for the appropriate exposure interval. The film was developed in a "Kodak X-Omat Processor Model M7A". Details are given elsewhere (Kettman *et al.*, 1986).

Image analysis

Radiofluorographic images were scanned by a "Molecular Dynamics" laser scanner and the images submitted to "Kepler" image analysis system (LSB, Rockville, USA) for spot modelling and spot matching.

The original radiofluorography image information is converted into so-called spot lists, in which each spot is defined by 5 bytes of information (2 bytes for the coordinate position x and y, and another 3 bytes for the spot volume [sx, sy, ampl]). Typically, a spot list containing say 600 spots will consist of $5 \times 600 = 3{,}000$ byte information. The original "pixel by pixel" information of the scanned image consisted of $1{,}700 \times 1{,}700 = 3{,}000{,}000$ bytes. Note that the spot list information is defined by three orders of magnitude less bytes than the scanned data. After completion of the image processing, one of the spot lists is chosen as a matrix to which all other spot lists will be compared. Such a list, to which the software program assigns a numbering system, is called master list or master pattern; all spot patterns will then be matched to the master pattern and individual spots will receive numbers congruent to the master spots. In this paper, the master list for liver samples (ML) and the master list for serum samples (MS) have independent numbering systems.

For the evaluation of spot intensity, radiofluorographs with properly adjusted exposure intervals were used so that a quantitative comparison of spot intensity would be manageable. The relative values are valid throughout spot comparisons shown in this paper.

Histograms

Two types of histogram are displayed: firstly, comparison of absolute spot volumes for a given spot pair is shown, and secondly, comparison of spot pairs, in which the larger of the pair is adjusted to max y value, is shown. Such histograms are online features of the Kepler software system. All database information described in this paper is available from I. Lefkovits upon request.

RESULTS

Mortality and MHV3 growth in infected BALB/c mice

BALB/c mice infected with MHV3 were shown to be fully susceptible to virus infection (100 % mortality) and, as can be seen in figure 1, the virus titres in the liver of infected mice increased gradually to a peak of 8×10^5 PFU/g of tissue after 3 days' infection, when the mice died of acute hepatitis.

2D-SDS-PAGE of liver preparations of MHV3-infected BALB/c mice

In figure 2, the 2D-SDS-PAGE spot pattern of liver preparations from non-infected (A) and from MHV3-infected mice (B) is shown, as well as the master pattern (ML), which is based on the non-infected liver preparation spot pattern. The 2D-SDS-PAGE of the non-infected liver preparation revealed 276 spots. Upon matching, 187 spots were found to be present in both patterns. The master pattern, when created, contained 225 spots of the non-infected liver

preparation, and then 32 spots from the infected liver preparation were added. Although all 89 spots could have been included in the master pattern, our conservative approach was to add only those spots which were reliably modelled.

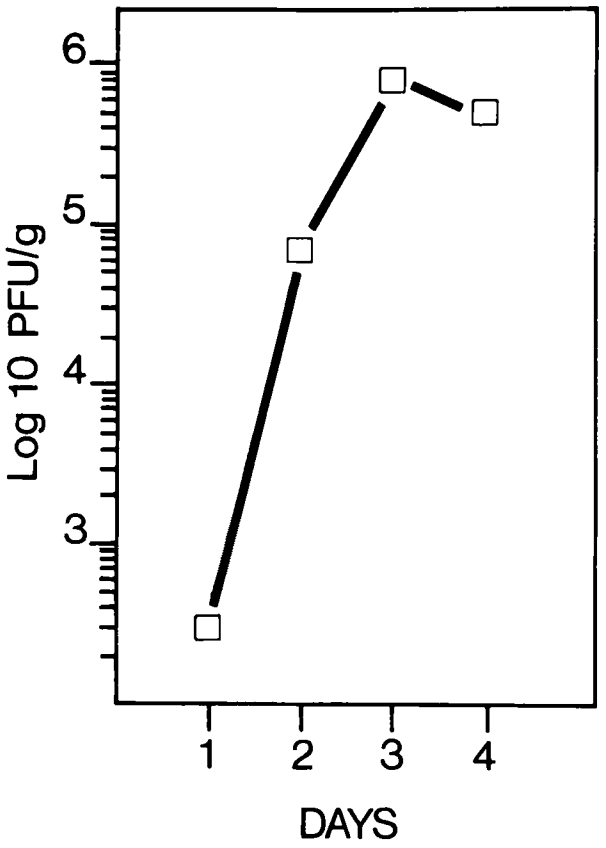


Fig. 1. Virus titres detected in the liver of MHV3-infected BALB/c mice.

Animals were i.p. inoculated with 10^3 PFU of MHV3. At subsequent intervals, groups of 5 mice were sacrificed, the liver obtained and the virus titrated. The MHV3 titres, reported as $\log_{10}$ PFU/g of liver, are the average of 5 different determinations.

In panel (a) of figure 2, all spots are depicted, the selected ones for panel (b) being highlighted. We have chosen and highlighted close to 60 spots, which are shown in panel (b) with their master number.

For all these selected spots, histograms of spot intensity are compiled in figure 3, showing the absolute spot volumes (panel I) and "max value"-adjusted spot ratios (panel II). Spots 20 and 70 are constant spots, and spots 26, 69, 92 and 198 show little variation in either direction. In tables I and II, spots which increase or

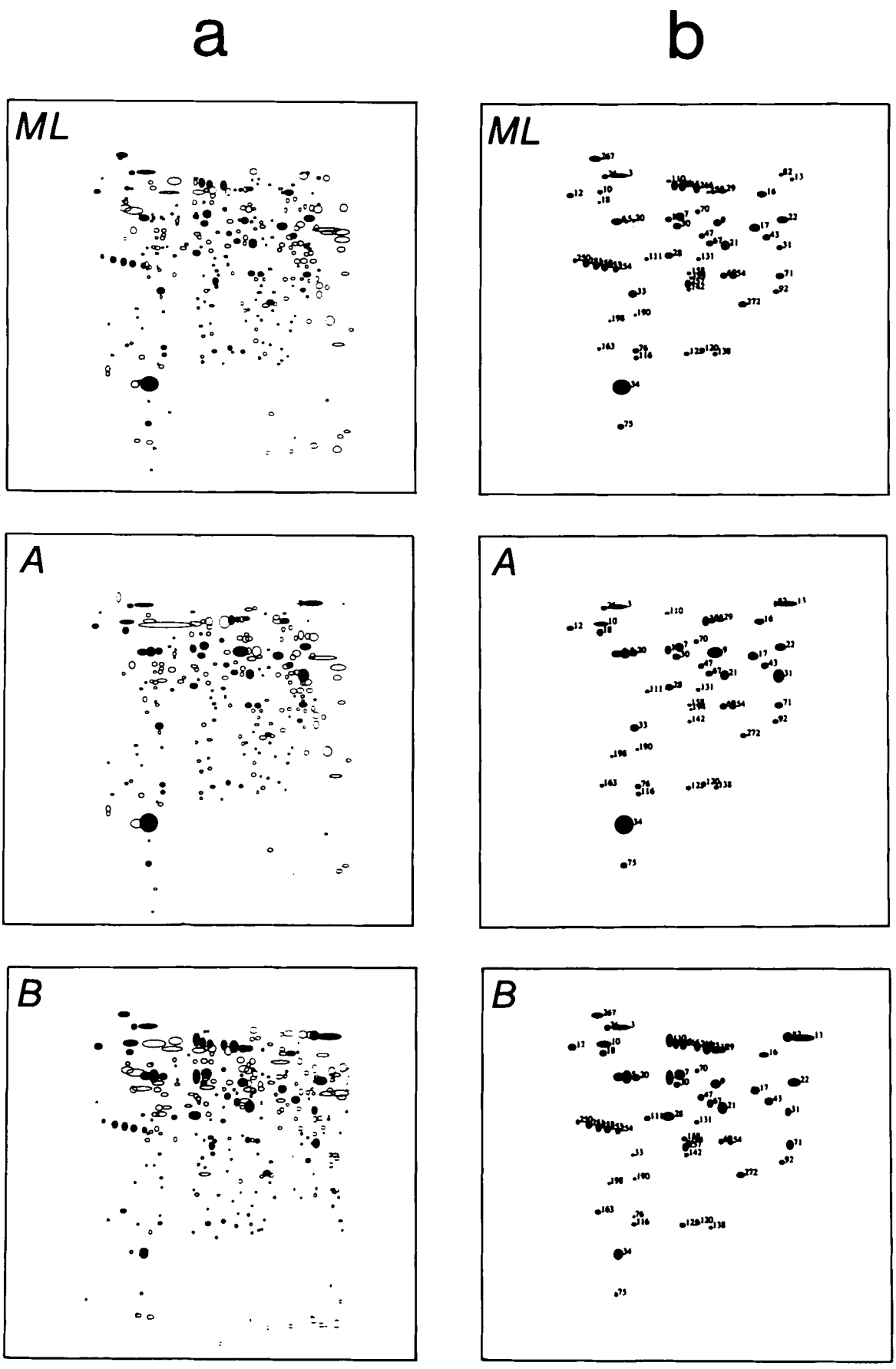
Table I. Increase in the abundance ratio of selected spots obtained by image analysis of 2D-SDS-PAGE of liver preparations from non-infected (A) or MHV3-infected (B) BALB/c mice.

Spot	Volume A	Volume B	Ratio of increase upon infection
10	12765	31950	2.50
12	11554	22146	1.92
14	18996	42885	2.26
25	10555	26717	2.53
28	20334	48715	2.40
38	7344	20553	2.80
43	14228	28545	2.01
47	8118	16502	2.03
67	5449	15584	2.86
71	8411	26322	3.13
82	3988	30666	7.69
110	1739	24821	14.27
111	2796	7182	2.57
158	1467	3361	2.29
163	1553	5602	3.61
194	782	2347	3.00
272	5343	18284	3.42

The ratio increase was calculated by volume comparison (arbitrary units).

Fig. 2. Image analysis of 2D-SDS-PAGE of liver preparations of non-infected (A) or MHV3-infected (B) BALB/c mice.

A mouse was inoculated i.p. with 10^3 PFU of MHV3 and two days later was injected i.v. with 1 mCi of ^{35}S-methionine and sacrificed after 4 h. The liver was obtained, solubilized and submitted to 2D-SDS-PAGE. Panel (a) displays the whole pattern with a subset of highlighted spots. Panel (b) displays the highlighted spots only, the spot numbers are displayed as well. ML = master pattern for the liver sample.


a
b
ML
ML
A
A
B
B

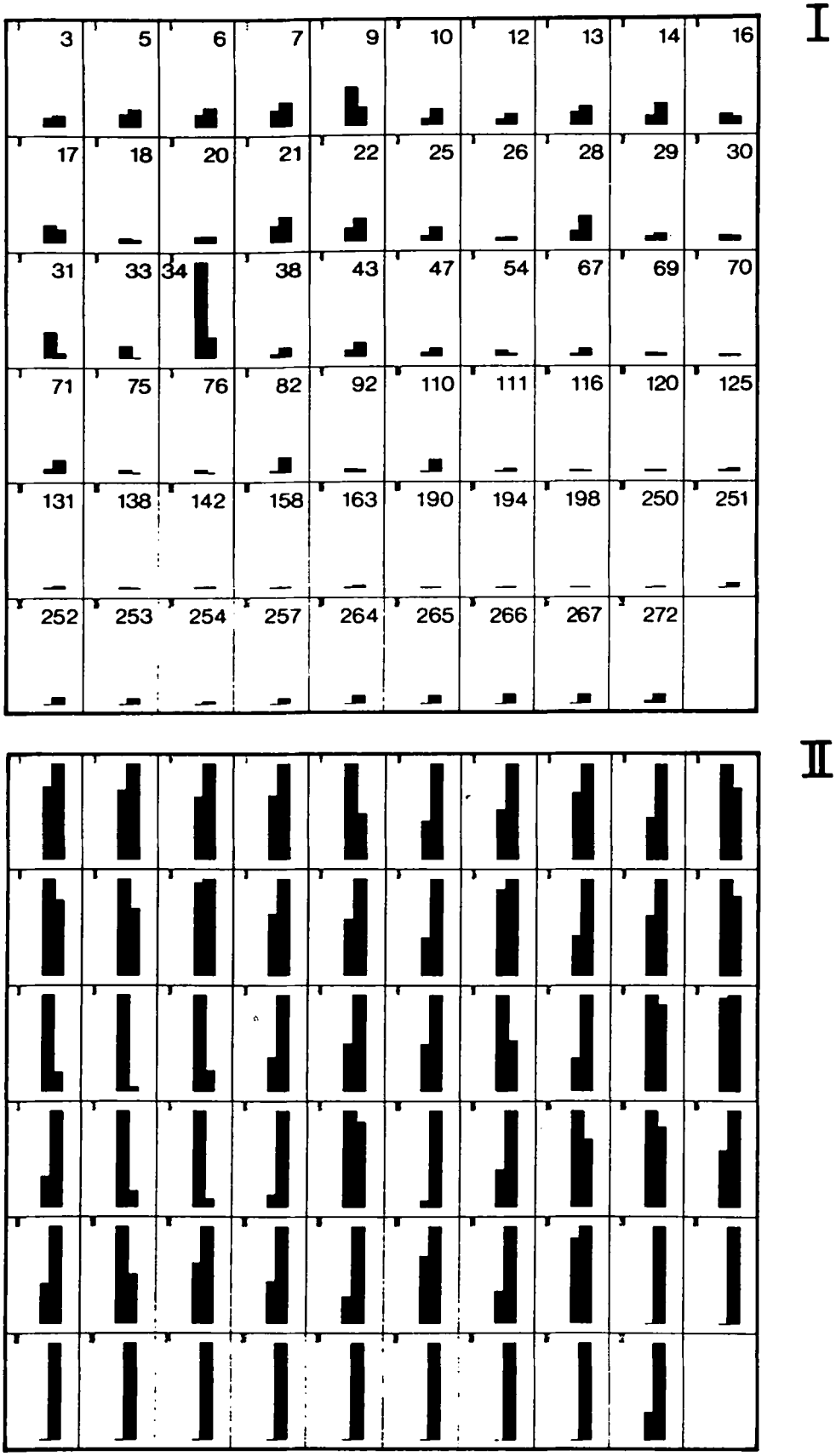


Fig. 3. Histograms of spot intensities.
In panel I, spot pairs are shown scaled separately for each spot pair. In panel II, a common scale for all spots is applied.

Table II. Decrease in the abundance ratio of selected spots obtained by image analysis of 2D-SDS-PAGE of liver preparations from non-infected (A) or MHV3-infected (B) BALB/c mice.

Spot	Volume A	Volume B	Ratio of decrease upon infection
9	75160	35908	2.09
31	50697	10117	5.01
33	23823	1157	20.59
34	186109	40750	4.57
54	12374	6579	1.88
75	7435	1277	5.82
76	7126	626	11.38
138	2985	1526	1.96

The ratio decrease was calculated by volume comparison (arbitrary units).

decrease in abundance by a factor of 2 or more are indicated. Furthermore, there is a set of spots which appears only in the pattern from the infected liver preparation, many of which are known to be serum constituents.

2D-SDS-PAGE of serum of MHV3-infected BALB/c mice

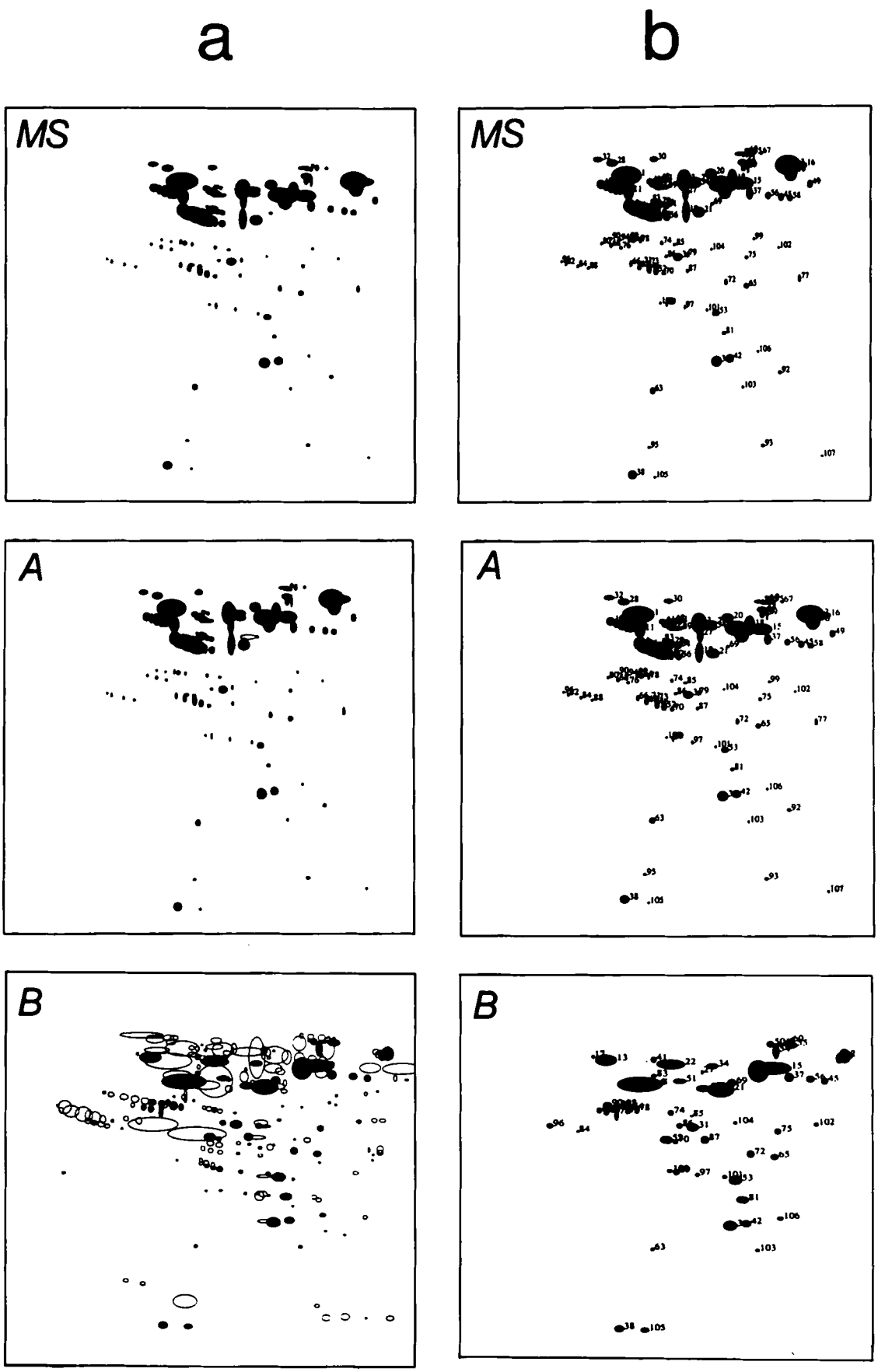
In figure 4, the spot patterns of serum of non-infected (A) and MHV3-infected (B) mice are shown. There are a large number of spots which are profoundly altered upon MHV3 infection. At present, no complete computer evaluation permits reliable modelling of closely spaced glycosylated spots. Thus, at present, it is worth stating that several spots from the liver samples of MHV3-infected animals may be serum spots. In a conventional 2D gel approach (*i.e.* without labelling of the entire mouse), serum spots do not interfere since they are not labelled and hence are not revealed on the radiofluorography.

DISCUSSION

The aim of the work presented here was to identify changes in liver protein expression upon infection of susceptible mice with MHV3. A powerful and also convenient procedure for identifying such changes is the labelling of the entire mouse with ^{35}S-methionine during the course of infection. The experiment requires a relatively high dose of ^{35}S-methionine which, as described earlier (Pluschke and Lefkovits, 1984), consists of injecting 1 mCi of ^{35}S-methionine i.v. into a mouse. Such treatment provides a pulse of radioisotope for the whole organism, and all the relevant biosynthetic processes make use of the provided radioisotope. The consequence is that not only do the cells become efficiently labelled but also the body fluid will contain proteins which were labelled intracellularly and then secreted into the extracellular space. Obviously the labelled polypeptides will be considerably diluted when secreted into the body fluids and will be detectable only above certain threshold concentrations. One hour after i.v. administration of ^{35}S-methionine, sufficient labelling was achieved so that a 2D gel exposed for one month revealed several hundreds of spots on a radiofluorograph.

Nevertheless, *in vivo* labelling imposed certain limitations upon the analysis; we did not detect 1,000-1,500 spots, as is the case for *in vitro* labelling but, as mentioned above, only about 300 spots. The label introduced into the animal is diluted in the body fluid (estimated volume 5 ml) and in the cytoplasmic content upon uptake into the cell, and the specific activity of the label is profoundly decreased by the rich amino acid pool of the animal. Thus, only those proteins which are synthesized at the highest rate will incorporate enough label to yield detectable spots. Nevertheless, most of these 300 spots could not be detected by any other means than by this methodology. Fine changes can be revealed only by *in vitro* labelling.

Infection with MHV3 induced profound alterations in the spot pattern of proteins synthesized in the liver of BALB/c mice. We found that 17 major proteins increased their rate of synthesis by a factor of 2 to 20 (tables I and II). The abovementioned figures refer to an alteration in the rate of synthesis and/or post-translational modification rather than the overall abundance.


a
b
MS
MS
A
A
B
B

Some spots from the liver sample are probably serum spots, as is the case for spot 110 which shows a 14-fold increase upon infection. It is advantageous to also consider the serum spots, since they may be in an as yet unknown fashion correlated to the events monitored in the liver.

Using the quantitative 2D-SDS-PAGE approach, we were able to obtain a new insight into the events after MHV3 infection, since our analysis was not restricted to following only one variable but rather to simultaneously monitoring all changes in the chosen sample. We identified and tagged 17 gene products which increased and 8 gene products which decreased in liver preparations upon MHV3 infection of BALB/c mice. In the near future, we shall attempt to identify some of these proteins by cDNA obtained from the gene catalogue of Lefkovits *et al.*, 1990, and we shall try to refine the above-described approach by analysing cell-free translation products from the mRNA obtained from infected and non-infected mice. This would lead us to the understanding of more refined regulatory events during the course of infection.

We have attempted to complete this study by evaluating the changes in the protein pattern (upon virus inoculation) of resistant mice (A/J). Preliminary experiments show that certain spots detected in BALB/c mice are not present in A/J mice. Since the pattern from intact (non-inoculated) A/J mice differs from the BALB/c pattern, rigorous evaluation will be possible only when there is an understanding of the genetic background differences (under study now).

We interpret the protein modification as the result of a defence attempt by the animal, perhaps by the formation of stress proteins. We hope that blotting (or alternatively immunoprecipitation and co-electrophoresis of the eluate) with defined antisera will shed some light on the nature of this response.

Acknowledgements

M.A.L. was a recipient of the Deutscher Akademischer Austauschdients (DAAD) during this research. The Basel Institute for Immunology was founded and is supported by F. Hoffmann-La Roche and Co. Ltd., Basel, Switzerland.

Importantes modifications des protéines synthétisées par le foie des souris sensibles à l'infection par le virus 3 de l'hépatite murine

Des souris BALB/c infectées expérimentalement par le virus 3 de l'hépatite murine voient leur titre en virus croître progressivement jusqu'à 8×10^5 PFU/g de tissu 3 jours après l'infection, moment où les souris meurent d'hépatite aiguë. Après l'infection par le virus, les souris ont été marquées *in vivo* par la ^{35}S-méthionine et des préparations de foie des animaux infectés et des animaux témoins ont été soumises à une électrophorèse bidimensionnelle. L'analyse comparée des images à l'aide d'un ordinateur a révélé que 17 protéines étaient détectées en plus grande quantité, alors que 8 autres étaient moins abondantes chez les souris infectées. Nous concluons que, pendant l'infection par le virus, les animaux sensibles présentent d'importantes perturbations des synthèses protéiques. Ces altérations concernent les protéines produites par la souris et non des produits de synthèse du virus. Il reste à déterminer si ces altérations sont le résultat d'une défense de l'animal ou si elles sont dictées par le virus afin d'enrayer une réponse de l'hôte.

Mots-clés: Coronavirus, Hépatite murine, Protéine; Synthèse, Foie, Marquage *in vivo*, Défenses.

Fig. 4. Image analysis of 2D-SDS-PAGE of serum of non-infected (A) or MHV3-infected (B) BALB/c mice.

A mouse was inoculated i.p. with 10^3 PFU of MHV3 and two days later was injected i.v. with 1 mCi of ^{35}S-methionine and sacrificed after 4 h. The serum was obtained, solubilized and submitted to 2D-SDS-PAGE. Panel (a) displays the whole pattern with a subset of highlighted spots. Panel (b) displays the highlighted spots only, the spot numbers are displayed as well. MS = master pattern for the serum sample.

References

Allen, R.C., Bienvenu, J., Laurent, P. & Suskind, R.M. (1982), Marker proteins in inflammation. Walter de Gruyer, Berlin.

Arnheiter, H., Baechi, T. & Haller, O. (1982), Adult mouse hepatocytes in primary monolayer culture express genetic resistance to mouse hepatitis virus type 3. *J. Immunol.*, 129, 1275-1281.

Bang, F.B. & Warwick, A. (1960), Mouse macrophages as host cells for the mouse hepatitis virus and the genetic basis of their susceptibility. *Proc. natl. Acad. Sci.* (Wash.), 46, 1065-1075.

Dupuy, J.M., Dupuy, C. & Decarie, D. (1984), Genetically determined resistance to mouse hepatitis virus type 3 is expressed in hematopoietic donor cells in radiation chimeras. *J. Immunol.*, 133, 1609-1615.

Eaton, J.W., Brandt, P. & Mahoney, J.R. (1982), Haptoglobin: a natural bacteriostat. *Science*, 215, 691-693.

Kettman, J.R., Kuhn, L., Young, P. & Lefkovits, I. (1986), Parameters of the labelling of mitogen-activated murine lymphocytes by ^{35}S-methionine for two-dimensional gel electrophoresis. I. — Effect of culture conditions. *J. Immunol. Methods*, 88, 53-57.

Kuschner, I. (1981), The acute phase reactants and the erythrocyte sedimentation rate, *in* "Textbook of rheumatology" (W.W. Kelley, E.D. Harris, S. Ruddy & C.B. Sledge) (p. 669). W.B. Sanders, Philadelphia.

Kuschner, I., Volanakis, J.E. & Gewurz, H. (1982), C-reactive protein and the plasma protein response to tissue injury. *Ann. N.Y. Acad. Sci.*, 389.

Lefkovits, I., Young, P., Kuhn, L., Kettman, J., Gemmell, A., Tollaksen, S., Anderson, L. & Anderson, N. (1985), Use of a large-scale two-dimensional ISODALT gel electrophoresis system in immunology, *in* "Immunological methods, Vol. 3" (I. Lefkovits & B. Pernis) (p. 163). Academic Press, New York.

Lefkovits, L., Kettman, J.R. & Coleclough, C. (1990), A strategy for founding a global lymphocyte proteinpaedia and gene catalog. *Immunol. Today*, 11, 157-162.

Levy, G.A., Leibowitz, J.L. & Edginton, T.S. (1981), Induction of monocyte procoagulant activity by murine hepatitis virus type 3 (MHV3) parallels disease susceptibility in mice. *J. exp. Med.*, 154, 1150-1157.

Lucchiari, M.A. & Pereira, C.A. (1989), A major role of macrophage activation by interferon gamma during mouse hepatitis virus type 3 infection. — I. Genetically dependent resistance. *Immunobiol.*, 180, 12-22.

Lucchiari, M.A. & Pereira, C.A. (1990), A major role of macrophage activation by interferon gamma during mouse hepatitis virus type 3 infection. — II. Age-dependent resistance. *Immunobiol.*, 181, 31-39.

Martin, J.P., Koehren, F., Rannou, J.J. & Kirn, A. (1988), Temperature sensitive mutants of mouse hepatitis virus type 3 (MHV3): isolation, biochemical and genetic characterization. *Arch. Virol.*, 100, 147-160.

O'Farrell, P.H. (1975), High resolution two-dimensional electrophoresis of proteins. *J. biol. Chem.*, 250, 4007-4021.

Pereira, C.A., Steffan, A.M. & Kirn, A. (1984a), Interactions between mouse hepatitis viruses and primary cultures of Kupffer and endothelial liver cells from resistant and susceptible inbred mouse strains. *J. gen. Virol.*, 65, 1617-1620.

Pereira, C.A., Mercier, G., Oth, D. & Dupuy, J.M. (1984b), Induction of natural killer cells and interferon during mouse hepatitis virus infection of resistant and susceptible inbred mouse strains. *Immunobiol.*, 166, 35-42.

Pluschke, G. & Lefkovits, L. (1984), Two-dimensional gel pattern of proteins synthesized *de novo* in lymphoid organs of the mouse. *Clin. Chem.*, 30, 2042-2047.

Pluschke, G., Jenni, L., van Alphen, L. & Lefkovits, I. (1986), Complete two-dimensional gel electrophoresis pattern of *de novo* synthesized acute phase reactants. *Clin. exp. Immunol.*, 66, 331-339.

Tollaksen, S.L., Anderson, W.L. & Anderson, N.O. (1981), Operation of the ISODALT system, report ANL-RIM-81-1, 6th ed., Argonne National Laboratory, Argonne, IL.

Virelizier, J.L. & Gresser, I. (1978), Role of interferon in the pathogenesis of viral diseases of mice as demonstrated by the use of anti-interferon serum. — V. Protective role in mouse hepatitis type 3 infection of susceptible and resistant strains of mice. *J. Immunol.*, 120, 1616-1619.

Weinberg, E.D. (1984), Iron withholding: a defense against infection and neoplasia. *Physiol. Rev.*, 64, 65.