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Evaluation of the inactivation of HIV-1 in human femoral heads by heat treatment to 80°C in a heating device (Lobator SD-1, Telos GmbH, Marburg,Germany).

Introduction

When large holes or defects have to be resolved in orthopedics and surgical traumatology reconstitution of bone is hampered by the availability of bone pieces to be transplanted. The selection of choice is a homograft. This kind of reconstitution is possible when small bone pieces are needed and can be taken, for example, from the ridge of the pelvis. Larger defects have to be filled by ceramics or by an allograft. As with blood transfusion, application of foreign material harbors the risk of infectious disease transmission, which can be reduced by proper donor selection but is not completely eliminated.

Bone transplantation is associated with the risk of transmission of different bacteria, like staphylococcus, pseudomonas, enterobactericeae and treponema pallidum that are efficiently destroyed reaching a temperature of more than 60°C. Protozoal infectious agents like trypanosoma and plasmodia are destroyed by heat exceeding 65°C. Bacterial spores will not be destroyed by moderate heat treatment. Relevant viruses transmitted by blood and organ transplants are cytomegalovirus, hepatitis B virus, hepatitis C virus, human T-leukaemia virus and human immunodeficiency virus. Their risk of transmission can be reduced by appropriate testing either of antibodies directed against the infectious agents or by detection of viral antigen. Only in hepatitis B virus infection is enough antigen present during seroconversion in blood allowing for the identification of infected, i.e. antigen positive, but still not antibody-carrying donors. The detection of early antigen levels is for technical reasons only occasionally possible in HIV and HCV infected individuals. The period in which a person is infected and infectious but not identified by the currently used antibody assays is called the window period. Infectiousness during this period can be overcome by proper inactivation of the transplant.

Objective

In this study artificial loading of femur heads with highly concentrated HIV was performed to measure the kinetic activity of reduction of the infectivity with increasing temperature. The objective of this procedure was to prove whether infectivity of HIV might be reduced by the heating process and to what extent, and to give information on the resting risk of HIV transmission by transplanted bone inactivated by the described procedure.

Material and Methods

The study was conducted and all experiments performed in accordance with Good Laboratory Practice.

Bone material

Capita femoris taken from patients undergoing artificial joint replacement were kept at -20°C until use.Articular cartilage was removed from each femur.The heads had a diameter between 4 and 5.5 cm. The bones had never been heated to higher temperatures before. Their internal spongiosa from the neck of the femoral head had been drilled out to allow a 100 µL PCR tube to be inserted, so that the center of the tube was located in the center of the femur head. PCR is polymerase chain reaction - thin walled tubes had been constructed for this procedure to allow easy energy transfer, and to keep the tubes closed from temperature ranges of 4°C to 95°C. For each of the inactivation experiments a new femoral head was used, totalling to 8

After placing the PCR tube in the hole a small disc was inserted to cover the lid of the tube. Onto the disc a silicon mass was polymerized to prevent any access of liquid. The silicon seal had a diameter of 1.5 cm and a depth of about 1.5 cm, depending on the size of the bone.

Heating apparatus

The Lobator SD-1 apparatus was an instrument supplied with a routine computer program for heating. The bone was deposited into a polypropylene container supplied by the company, 250 mL sterile Ringer-Solution added, and the heating program started with continued stirring of the Ringer-Solution.

Cooling to room temperature was performed as recommended by continuing stirring the Ringer Solution at ambient temperature to 20 to 40 °C with the magnetic stirrer of the apparatus. Heating temperature was controlled on the indicator screen on the front panel of the instrument, the temperature decline during the cooling process was roughly monitored by checking with the finger.

To analyze the kinetic activity of the HIV decay, the heating process was interrupted at selected times. This was done in one run after 5, 20 and 40 min, and in the second run after 10, 30 and 50 min. The 5 min value was taken as a bone control, to be independent of the culture control, which was the titration of the HIV from the stock solution prepared (control 1 and control 2).

Virus

The HIV-1 IIIB virus was choosen for these experiments in order to have some kind of standardization. The IIIB virus is named today LAI and was initially supplied to the Pettenkofer Institute in 1984 by R.Gallo, NIH, Bethesda. The virus was grown on HUT-78 cells in RPMI 1640 supplemented with 10% fetal calf serum and the antibiotics penicillin and streptomycin, termed as medium. Virus titer in the cell supernatant which was exchanged on a weekly basis was $10^{5.5}$ TCID50 per mL. Cell concentration was 10^6 per mL.

Virus stock for the inactivation experiments was prepared by spinning 10 mL of a culture cell suspension for 7 min at 1700 x g , removing 9 mL of the supernatant, and suspending the sedimented cells in the resting supernatant. Of this suspension 100 µL were filled in a PCR tube and inserted in the drilled hole of the femur head. The hole was sealed as described above.

Titration

To quantify the virus content microtiter plates were used and the cell and virus suspension titrated in 10 fold dilutions. Since only 100 µL of the tube content were available, 10 µL were added to each of the 8 wells of the first row of the microtiter plate, thoroughly mixed with a multichanel pipette and 10 µL transferred to the wells of the second row which contained again 90 µL medium. End point dilutions were made for the first 30 min of the inactivation procedure to 10 and 9 dilution steps, and were reduced for the 40 to 62 min inactivation steps. Microtiter plates were from Nunc, Wiesbaden.

After titration of the virus suspension 50 µL HUT-78 cells were added to each well, as a suspension of 10^6 cells per mL medium. Each microtiter plate was covered by a lid and handled individually and incubated for 21 days in a 6% CO_2 atmosphere at 36°C. 10 µL of the supernatant were removed for the determination of p24-antigen after 1, 8 , 16 and 21 days in a 1:50 dilution.

Quantitative evaluation was done according to method of Kärber and Sperman after the determination of the p24-antigen content in the supernatant of the cells. Extinctions >1.5 were finally considered as positive.

Detection of viral growth

There are two ways to monitor virus growth: one by giant cell formation and the other by the determination of p24-antigen in the supernatant with a comercially availble ELISA.

Giant cell formation was read twice weekly by using an inverted microscope. Freshly occuring syncytia can be nicely distinguished and followed over time. Since also the inactivated cell suspension added to the PCR tubes contained giant cells, which were not destroyed by the inactivation process, the reading of the first two rows of the microtiter plates was partially difficult.

p24-antigen detection: the assay from Pasteur, Marne la Coquette in France was used, which is run in

microtiter plates. The procedure of the manufacturer was follwed, but to avoid measuring background viral antigen, which might originate from dead virus as well, a 1 in 50 dilution of the supernatant was used. From each well the determination of the p24-antigen was performed individually - there was no pooling - and over time as indicated above. ELISA extinctions were read on a photometer at 492 nm as recommended. Only extinctions > 1.5 were finally considered as positive to avoid any detection of dead virus material resting in the cell supernatant or being liberated during time from the dead cells transferred to the wells of the microtiter plate from the PCR tube.
The code of the antigen assay was 72236 and the lot number 5F048.R . Expiration date was 5 months ahead of the use of the assay.

To assure that in the first row of the 40 min and 62 min inactivation protocol there was no HIV growth, the resting content of the wells, i.e. cells and supernatant were aspirated after 28 days of culture and transferred to the wells of a 48 well microtiter plate, which each contained 200µL medium and 10^5 HUT-78 cells. Within these wells virus growth was checked either by the occurence of giant cells, which did not occur, and by the quantification of p24-antigen, which also did not increase.

Results

HIV control titer

The initial HIV-cell suspension was titrated for the two inactivation experiments. The titer obtained was 10 log 10.12 and 10 log 10.38. As may be seen from Figure 1 and 2 the maximal titer was reached at 16 days of culture and did not rise further to day 21, indicating that titers were determined at the endpoint of viral growth.

Titer decrease during heat inactivation

Two runs were performed to determine the decay of HIV infectivity during the inactivation procedure.
In the first experiment the heating process was interrupted at 5, 10 and 40 min and the last femur head was processed to the recommended 62 min, when the heating apparatus is signaling that heating time has expired. In order to get a more detailed time scale of HIV inactivation a second experiment was done in which heating time was stopped after 10, 30 and 50 min. Final titers obtained after the titration experiments on day 21 of culture are given in Table 1. These data indicate that after 30 min of heat treatment there was viable HIV, which induced giant cells in one well. After 40, 50 and 62 min there was no longer virus growth detectable, therefore a titer of 0 is indicated. These reults are summarized in Figure 3.

Table 1

Inactivation of HIV-1 in femur head as a suspension of free virus and virus infected HUT-78 cells. Numbers given indicate the 10 log values obtained after x days of culture. 0 indicates no viral growth.

Experiment 1

Day of culture	control 1	5 min	20min	40 min	62 min
1	1.62	3.62	2.44	0	0
8	9.9	6.68	4.5	0	0
16	10.12	8.18	5.38	0	0
21	10.12	8.18	5.38	0	0

Experiment 2

day of culture	control 2	10 min	30 min	50min
1	4.19	4.0	0	0
8	8.06	6.25	0	0
16	10.38	7.44	1.93	0
21	10.38	7.44	1.18	0

These data indicate that HIV deposited into the center of the capita femoris was inactivated after 40 min heating time. The temperature reached at this time in the center of the bone should be > 68°C according to the data given by the manufacturer of the heating machine.

Subcultivation of cells and supernatant of the 40 and 62 min inactivation process
To check that within the first dilution of the titration from the time 40 min and 62 min in fact no HIV replication occured, the content of the wells from the first row of the microtiter plates - cells and supernatant - was transferred each to individual wells of a 48 well plate , 10^6 HUT-78 cells added and incubated. Within three weeks there was no viral growth, since no giant cell formation was visible and no increase of p24-antigen in the cell supernatant (values are given on page 15 and 18).
Individual values obtained with the p24-antigen assay are given in the annex on pages 7 to 26.

Discussion

While liquids or protein suspensions can be spiked with HIV and its inactivation followed by heat treatment or exposure to different chemicals, such as detergents, a homogeneous spiking of a bone piece is not possible. Penetration of chemical agents like detergent or ethanol in the spongiosa of the bone might be possible but from theoretical considerations seems to be imperfect in the cortical part of the bone (compacta). Therefore we have chosen a design by incubation of the femoral head within liquid, in which heat has to be transferred from the outer parts of the bone which contains the dense cortex into the inner part of the spongiosa where the tube with the HIV was positioned. The hole for inserting the tube was sealed with a thick layer of silicone to prevent heat transfer via this opening.

The results obtained should be interpreted with several remarks and from various aspects.
1. The HIV within the tube was cell bound and free and corresponds roughly to natural conditions in the body, where both forms are present. The titer obtained was more than 10^8, which is about 100 fold higher than the highest titer obtained under natural conditions in a patient (1).

2. The decay of HIV observed was continously falling, without showing a tailing effect. Tailing might have been seen when shorter time intervalls for checking the titer would have been chosen. Nevertheless the obtained titers allow some explanation. The 20 min value obtained is with $10^{5\,38}$ very close to the titer obtained in the cell supernatant which gave a value of $10^{5\,5}$. The temperature reached at that time in the center of the bone is calculated to be approximately 40°C. During the first minutes of cooling there might be a further increase of the temperature in the inner part of the bone which leads to destruction of the HUT-78 cells and indicates that the titer found corresponds to resting free virus. After 30 min of heating the temperature within the center of the bone had been given by the manufacturer to be approximately 60°C, a temperature that allows HIV survival for a short period of several minutes (2).Total inactivation of HIV might be prolonged by the high protein content in the PCR tube.The data obtained are in accordance with the

HIV decay at 56°C as described by Spire et al. (3).
In conclusion the data of the falling HIV titer during the increasing temperature confirms those results that had been obtained previously for HIV in liquids (2,3,4) and for HTLV (5).

3. The procedure followed in this protocol was done with the reference strain HIV-1 IIIB. Since between HIV-1 and HIV-2 inactivation there had been no differences found in plasma treated with heat it can be assumed that variant HIV's that are not properly detected by the currently used assays are also inactivated in the femoral head by this heating process.

5. The p24-antigen assay detects this inner core protein of HIV from live and dead virus. The initial virus supension had a titer of 10^{10}, corresponding to 1000 ng of p24-antigen. The limit of detection of the assay is 10 pg, that means it will detect one hundredthousandth part of the suspension. This was the reason to recultivate the titrations from the 40 min and 62 min values and this was further the reason to take the obtained p24-antigen values only as positive when an extinction of >1.5 was measured.
The p24-antigen assay detects additionally the inner core protein from cell bound virus, when dead cells of the spiking suspension had been aspirated performing the weekly check for virus growth. Due to this high overflow amount of p24-antigen in the first rows of the mictrotiter plates, extinctions measured were only recognized as reliable when they increased over time.

The design of this study was to check whether the HIV inserted artificially into the bone could be inactivated by the applied heating process to finally 80°C. After 40 min of treatment reaching a temperature of >68°C no HIV growth could be detected with the method described. The conclusion from this finding is that first for the next 22 min of the heating process there should be enough time left to destroy any additional HIV which might be present or when the femoral head has a bigger size than those used in these experiments, which is a supplemental safety factor. Secondly,as described above, the observed HIV destruction coincides well with previously described results of the sensitivity of HIV to heat treatment (2,3).

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Prof.Dr.L.Gürtler

Prof.Dr.G.Ruckdeschel

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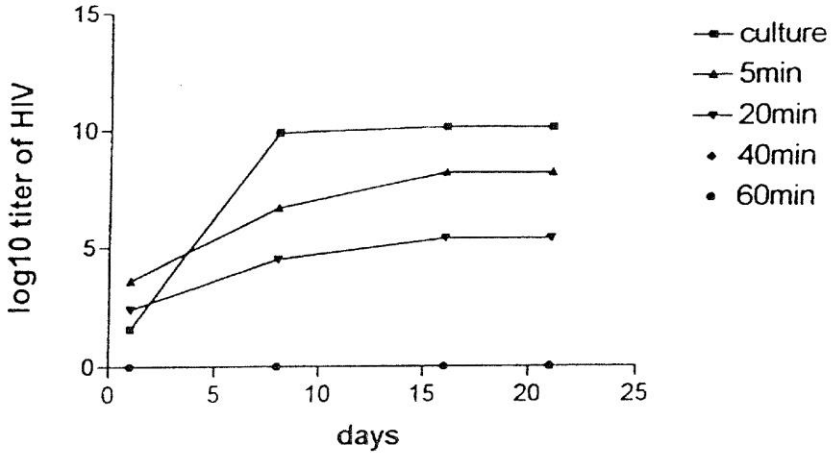

Figure 1

Kinetics of the HIV replication by titration in microtiter plates after treating in a automatic heater and interrupting the heating process after 5, 20, 40 and 62 min. HIV and the HUT-78 infected cells were seeded out in 10 fold dilutions and after addition of 10^5 uninfected HUT-78 cells the HIV growth monitored by the formation of giant cells (not shown) and the determination of p24-antigen in the cell supernantant. Calculation of the titer was done according to the method of Sperman and Kärber. As can be seen the maximal titer was already reached after 16 days of incubation.

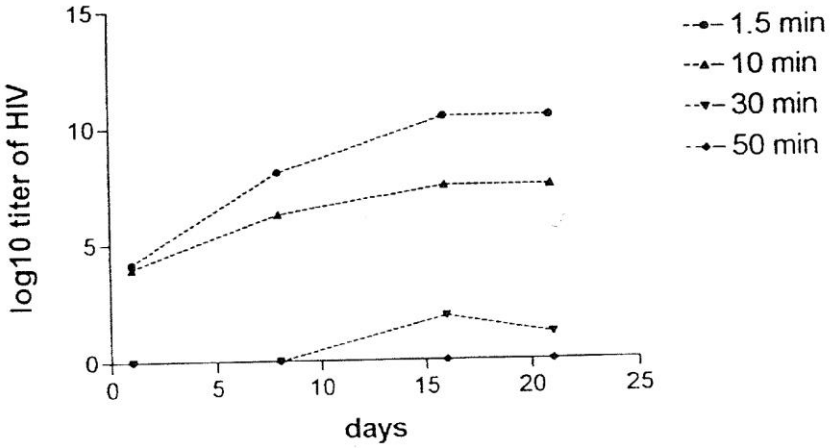

Figure 2

Kinetics of the HIV replication by titration of the heat exposed cell suspension within the femur head and interruption of the process after 10, 30 and 50 min. Further details are described in the legend to Figure 1. As indicated in this figure a low titer was obtained after 30 min heat exposure, while after 50 min no viral growth was detected. Within the 30 min values a decrease of the titer from $10^{1.93}$ to $10^{1.18}$ after 21 days was observed. As mentioned in the discussuion first row values in the micotiter plate might give contaminations to false positive p24-antigen results. Since in one of the wells the formation of giant cells was seen, the obtained titer is at least $10^{1.18}$.

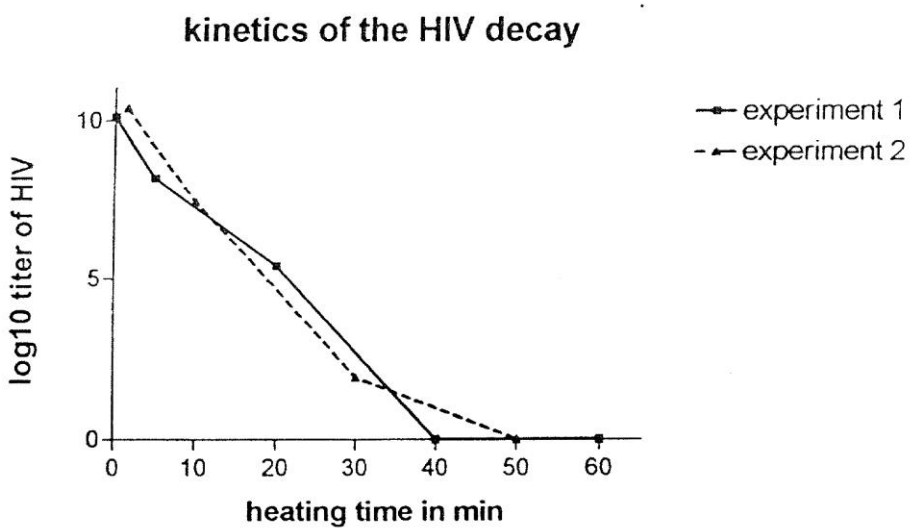

Figure 3
Kinetics of the HIV decay over time after exposure to the heating process to finally 80°C. The first values at zero time are those of the original HIV control titration. In experiment 1 heating process was stopped after 5, 20, 40 and 62 min, in experiment 2 after 10, 30 and 50 min. No HIV growth was seen after exposure to heat after 40 min and longer. After 40 min a temperature in the center of the bone of approximately 68°C or higher is reached according to the preevaluations made for the instrument.

Annex

p24-antigen extinctions obtained for the different time points at days 1, 8, 16 and 21 are given, measured at 492 nm. 2.5 means overflow extinction, i.e. > 2.5

HIV control suspension 1
day 1, HIV control 1

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10 dilution		
2.5	2.5	2.5	2.5	0.86	0.27	0.14	0.09	0.23	0.10	0.13	2.5
2.5	2.5	2.5	2.5	0.98	0.30	0.12	0.08	0.17	0.08	0.11	2.5
2.5	2.5	2.5	2.5	1.18	0.31	0.12	0.08	0.11	0.09	0.11	2.5
2.5	2.5	2.5	2.5	1.24	0.34	0.11	0.07	0.11	0.09	0.13	2.5
2.5	2.5	2.5	2.5	1.02	0.30	0.08	0.08	0.09	0.09	0.10	2.5
2.5	2.5	2.5	2.5	1.20	0.32	0.08	0.07	0.08	0.08	0.09	2.5
2.5	2.5	2.5	2.5	1.23	0.45	0.08	0.06	0.07	0.08	0.07	2.5
2.5	2.5	2.5	2.5	1.18	0.30	0.07	0.05	0.08	0.06	0.07	2.5
									neg control	pos	

day 8, HIV control 1

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10	neg control	pos control
2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.69	0.78	0.61	0.36	2.5
2.5	2.5	2.5	2.5	2.5	2.5	2.5	1.35	0.89	0.59	0.49	2.5
2.5	2.5	2.5	2.5	2.5	2.5	2.5	1.65	1.77	2.27	0.69	2.5
2.5	2.5	2.5	2,5	0.57*	2.5	1.30*	1.60	1.99	0.97	nv	2.5
2.5	2.5	2.5	2.5	0.86*	2.5	0.78*	2.5	0.54	0.29	0.40	2.5
2.5	2.5	2.5	2.5	0.31*	2.5	0.47*	1.65	2.48	0.15	0.19	2.5
2.5	2.5	2.5	2.5	0.11*	2.5	2.5	2.5	0.38	0.09	0.18	2.5
2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	1.63	0.86	nv	2.5

nv is no value, * is inappropriate sample dilution

day16, HIV control 1

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10	neg control	pos control
nd	nd	nd	nd	nd	nd	2.5	2.5	2.5	2.5	0.41	nd
						2.5	2.5	2.5	2.5	0.37	
						2.5	2.5	2.5	2.5	0.29	
						2.5	2.5	2.5	2.5	0.33	
						2.5	2.5	2.5	0.32	0.82	
						2.5	2.5	2.5	0.22	0.53	
						2.5	2.5	2.5	0.65	0.61	
						2.5	2.5	2.5	2.5	0.46	

nd is not done, since positive in the previous assay

day 21, HIV control 1

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
nd	nd	nd	2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.17	2.5
			2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.32	2.5
			2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.24	2.5
			2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.41	2.5
			2.5	2.5	2.5	2.5	2.5	2.5	0.25	0.30	2.5
			2.5	2.5	2.5	2.5	2.5	2.5	0.22	0.19	2.5
			2.5	2.5	2.5	2.5	2.5	2.5	0.18	0.24	2.5
			2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.28	2.5
										neg control	pos

Heat treatment for 5 min
day 1 HIV, 5 min inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
2.5	2.5	1.81	0.47	0.02	0.04	0.02	0.02	0.09	0.07		
2.5	1.60	0.51	0.12	0.04	0.02	0.03	0.01	0.10	0.03		
2.5	2.36	0.71	0.22	0.06	0.03	0.03	0.02	0.07	0.03		
2.5	1.82	0.47	0.15	0.05	0.03	0.02	0.01	0.05	0.03		
2.5	1.47	0.17	0.08	0.03	0.02	0.01	0.01	0.04	0.03		
2.5	1.65	0.14	0.08	0.02	0.01	0.02	0.01	0.01	0.02		
2.5	1.64	0.15	0.04	0.03	0.03	0.01	0.01	0.03	0.02	0.02	2.5
2.5	1.66	0.21	0.06	0.03	0.02	0.01	0.01	0.02	0.01	0.02	2.5
										neg control	pos

day 8 HIV, 5 min inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
2.5	2.5	2.5	2.5	1.18	0.03	0.05	0.02	0.03	0.02		
2.5	2.5	2.5	2.5	2.5	1.74	0.13	0.02	0.03	0.02		
2.5	2.5	2.5	2.5	2.5	2.5	1.17	0.02	0.02	0.02		
2.5	2.5	2.5	2.5	2.5	2.5	0.71	0.02	0.01	0.01		
2.5	2.5	2.5	2.5	2.5	1.86	0.24	0.01	0.02	0.02		
2.5	2.5	2.5	2.5	2.5	2.5	0.02	0.11	0.02	0.01		
2.5	2.5	2.5	2.5	2.5	0.04	0.35	0.01	0.01	0.01	0.02	2.5
2.5	2.5	2.5	2.5	2.5	1.78	0.17	0.72	0.01	0.01	0.02	2.5
										neg control	pos

day 16 HIV, 5 min inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
nd	nd	nd	nd	nd	0.19	0.32	0.26	0.32	0.16		
					2.5	2.5	2.5	0.48	0.08		
					2.5	2.5	2.5	0.18	0.08		
					2.5	2.5	0.33	0.20	0.08		
					2.5	2.5	0.18	2.5	0.14		
					2.5	2.5	2.5	0.24	0.07		
					2.5	2.5	0.18	0.07	0.09	0.28	2.5
					2.5	2.5	2.5	0.08	0.09	0.16	2.5
										neg control	pos

day 21, HIV 5 min inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
nd	nd	nd	2.5	2.5	0.28	0.34	0.20	0.20	0.11		
			2.5	2.5	2.5	2.5	2.46	0.18	0.20		
			2.5	2.5	2.5	2.5	2.5	0.24	0.13		
			2.5	2.5	2.5	2.5	0.49	0.27	0.19		
			2.5	2.5	2.5	2.5	0.52	2.5	0.24		
			2.5	2.5	2.5	2.5	2.5	0.26	0.22		
			2.5	2.5	2.5	2.5	0.23	0.27	0.19	0.29	2.5
			2.5	2.5	2.5	2.5	2.5	0.19	0.25	0.22	2.5
										neg control	pos

HIV inactivation in femur head
Heat treatment for 20 min

day 1, HIV 20 min inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
2.5	1.11	0.13	0.04	0.03	0.02	0.02	0.03	0.08	0.06		
1.73	0.33	0.05	0.02	0.03	0.02	0.03	0.02	0.07	0.03		
1.46	0.38	0.04	0.03	0.03	0.02	0.02	0.02	0.05	0.04		
2.5	0.70	0.13	0.03	0.03	0.01	0.02	0.02	0.08	0.03		
2.5	0.74	0.12	0.04	0.03	0.02	0.01	0.01	0.05	0.03		
2.5	0.78	0.07	0.03	0.02	0.01	0.01	0.01	0.02	0.02		
1.91	0.57	0.04	0.02	0.02	0.02	0.01	0.01	0.02	0.02	0.03	2.5
2.07	0.41	0.05	0.02	0.03	0.03	0.01	0.01	0.02	0.02	0.03	2.5
										neg control	pos

day 8, HIV 20 min inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
2.5	2.5	2.5	0.98	0.07	0.05	0.07	0.05	0.04	0.02		
2.5	2.02	1.18	0.21	0.07	0.04	0.04	0.03	0.04	0.01		
2.5	2.5	2.5	0.11	0.11	0.04	0.03	0.03	0.04	0.02		
2.5	2.5	2.5	2.5	1.82	0.44	0.05	0.02	0.02	0.01		
2.5	2.00	0.23	2.5	0.75	0.21	0.41	0.02	0.02	0.02		
2.5	2.5	2.5	2.5	0.53	0.02	0.01	0.02	0.01	0.01		
2.5	2.5	2.5	1.29	0.89	1.64	0.02	0.02	0.01	0.02	0.02	2.5
2.5	2.5	2.5	1.22	0.38	0.11	0.03	0.02	0.02	0.01	0.02	2.5
										neg control	pos

day 16, HIV 20 min inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
nd	nd	nd	2.5	2.5	0.53	0.14	0.12	nd	nd		
			2.5	0.41	0.46	0.18	0.11				
			2.5	2.5	0.16	0.16	0.12				
			2.5	2.5	2.5	0.17	2.5				
			2.5	2.5	2.5	2.5	0.13				
			2.5	2.5	0.17	0.21	2.5				
			2.5	2.5	2.5	2.5	0.14			nd	nd
			2.5	2.5	2.27	2.5	0.27			nd	nd
										neg control	pos

day 21, HIV 20 min inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
nd	nd	2.5	2.5	2.5	0.23	0.12	0.09	0.82	0.12		
		2.5	2.5	0.23	0.36	0.14	0.19	0.11	0.35		
		2.5	2.5	2.5	0.29	0.37	0.34	0.25	0.16		
		2.5	2.5	2.5	2.5	1.37	2.5	0.15	0.25		
		2.5	2.5	2.5	2.5	2.5	0.30	0.26	0.17		
		2.5	2.5	2.5	1.15	1.65	2.5	0.33	0.22		
		2.5	2.5	2.5	2.5	2.5	0.25	0.36	0.74	0.29	2.5
		2.5	2.5	2.5	2.5	2.5	0.76	0.60	0.56	0.21	2.5
										neg control	pos

HIV inactivation by heat treatment in bone

day 1, HIV 40 min inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
0.26	0.05	0.04	0.04	0.04	0.02	0.02	0.01	0.04	0.04		
0.26	0.03	0.03	0.02	0.03	0.01	0.02	0.01	0.04	0.02		
0.47	0.05	0.03	0.02	0.03	0.01	0.02	0.01	0.03	0.02		
0.26	0.03	0.03	0.02	0.03	0.02	0.01	0.02	0.03	0.02		
0.53	0.03	0.02	0.02	0.02	0.02	0.01	0.01	0.02	0.02		
0.54	0.06	0.03	0.02	0.02	0.02	0.01	0.01	0.02	0.02		
0.38	0.03	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.02	0.02	2.5
0.36	0.03	0.03	0.02	0.03	0.03	0.01	0.01	0.01	0.02	0.02	2.5
										neg control	pos

day 8, HIV 40 min inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
0.43	0.06	0.12	0.06	0.11	0.08	0.02	0.02	0.05	0.02		
0.28	0.04	0.10	0.05	0.09	0.04	0.03	0.02	0.06	0.02		
0.64	0.11	0.09	0.08	0.07	0.05	0.04	0.02	0.04	0.02		
0.41	0.06	0.07	0.05	0.06	0.28	0.02	0.02	0.02	0.02		
0.66	0.05	0.05	0.09	0.05	0.04	0.02	0.02	0.02	0.02		
0.60	0.11	0.04	0.03	0.04	0.03	0.02	0.05	0.02	0.02		
0.44	0.07	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.01	0.01	2.5
0.43	0.06	0.03	0.02	0.03	0.02	0.01	0.01	0.01	0.01	0.02	2.5
										neg control	pos

day 16, HIV 40 min inactivation

-1	-2	-3	-4
2.5	0.38	nd	nd
0.69	0.18		
0.79	0.37		
0.66	0.19		
1.01	0.23		
1.11	0.27		
0.87	0.17		
0.77	0.22		

day 21, HIV 40 min inactivation

-1	-2	-3	-4
2.5	0.19	0.11	nd
0.63	0.25	0.32	
1.14	0.34	0.29	
0.69	0.39	0.28	
1.20	0.41	0.14	
1.12	0.52	0.12	
0.75	0.37	0.13	
0.70	0.36	0.16	

day 28, HIV 40 min inactivation

-1	-2	-3	-4
2.5	2.5	2.5	nd
1.06	1.39	1.51	
1.27	1.59	1.86	
0.99	1.27	1.42	
1.44	1.51	2.35	
1.63	1.55	1.86	
1.13	1.46	1.47	
1.08	0.99	1.26	

Subtitration of the well content of the first row of day 24 in a new microtiter plate of the 40 min inactivation tiration

day 8	day 16	day 21
1.48	1.53	1.50
0.50	0.38	1.26
0.93	0.54	0.89
1.04	0.40	0.30
2.29	0.43	0.26
1.20	0.39	0.27
0.97	0.39	0.20
0.54	0.41	0.39

Heat treatment of HIV in bone

day 1, HIV inactivation 62 min

-1	-2	-3	-4	-5	-6	-7	-8	-9			
0.61	0.03	0.03	0.03	0.02	0.02	0.02	0.02	0.02		0.04	2.47
0.50	0.02	0.04	0.02	0.03	0.02	0.02	0.02	0.03		0.04	2.5
0.45	0.02	0.03	0.02	0.03	0.02	0.02	0.02	0.04		0.02	2.5
0.53	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.05		0.02	2.5
0.45	0.02	0.01	0.02	0.02	0.03	0.02	0.01	0.02		0.03	2.5
0.50	0.02	0.02	0.01	0.01	0.02	0.02	0.02	0.02		0.02	2.5
0.44	0.02	0.02	0.01	0.01	0.02	0.01	0.01	0.02		0.02	2.5
0.62	0.02	0.01	0.02	0.01	0.01	0.01	0.01	0.02		0.02	2.5
										neg control	pos

day 8. HIV inactivation 62 min

-1	-2	-3	-4	-5	-6	-7	-8	-9			
0.51	0.03	0.02	0.01	0.02	0.02	0.02	0.02	0.02		0.03	2.5
0.47	0.02	0.02	0.01	0.02	0.01	0.02	0.02	0.01		0.02	2.5
0.35	0.02	0.02	0.03	0.02	0.01	0.02	0.02	0.01		0.02	2.5
0.41	0.02	0.01	0.02	0.01	0.02	0.01	0.01	0.01		0.03	2.5
0.45	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01		0.02	2.5
0.43	0.01	0.07	0.01	0.01	0.01	0.01	0.01	0.01		0.02	2.5
0.50	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01		0.03	2.5
0.68	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.02	2.5
										neg control	pos

day 16, HIV 62 min inactivation

-1	-2	-3	-4
1.70	0.52	nd	nd
1.05	0.20		
0.97	0.18		
0.87	0.35		
1.03	0.21		
0.96	0.20		
0.82	0.17		
0.98	0.18		

day 21, HIV 62 min inactivation

-1	-2	-3	-4								
1.49	0.11	0.06	nd							0.11	2.5
0.83	0.10	0.07								0.16	2.5
0.52	0.11	0.07								0.09	2.5
0.63	0.18	0.09								0.08	2.5
0.73	0.08	0.09								0.08	2.5
0.63	0.36	0.10								0.09	2.5
0.76	0.08	0.15								0.11	2.5
0.75	0.11	0.17								0.26	2.5
										neg control	pos

Subtitration of the first row of the microtiter plate of the 62 min heat inactivation after 28 days of incubation on fresh HUT-78 cells to check for virus growth

day 8	day 16	day 21
1.82	1.69	2.19
1.33	1.44	1.29
0.94	1.53	1.04
1.21	1.12	1.07
1.07	1.16	1.19
1.06	1.00	1.10
1.13	1.14	1.10
1.93	1.31	1.74

HIV titration control 2

day 1, control 2

-1	-2	-3	-4	-5	-6	-7	-8	-9			
2.5	2.5	2.5	2.5	2.5	0.16	0.09	0.32	0.07			
2.5	2.5	1.97	0.82	0.46	0.22	0.13	0.17	0.08			
2.5	2.5	2.47	0.93	0.56	0.11	0.09	0.12	0.08			
2.5	2.5	2.27	0.85	0.79	0.18	0.15	0.20	0.08			
2.5	2.5	2.06	0.66	0.64	0.18	0.13	0.11	0.11			
2.5	2.5	1.90	0.65	0.39	0.36	0.11	0.13	0.13			
2.5	2.37	1.84	0.61	0.29	0.18	0.08	0.15	0.15		0.11	2.5
2.5	2.5	2.5	1.27	0.59	0.10	0.22	0.14	0.12		0.09	2.5
										neg control	pos

day 8, HIV control 2

-1	-2	-3	-4	-5	-6	-7	-8	-9			
2.5	2.5	2.5	2.5	2.5	0.58	0.90	2.5	0.58			
2.5	2.5	2.5	2.5	2.5	0.90	0.34	2.35	2.42			
2.5	2.5	2.5	2.5	2.5	0.65	2.5	2.5	2.5			
2.5	2.5	2.5	2.5	2.5	1.27	0.80	1.95	2.5			
2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5			
2.5	2.5	2.5	2.5	2.5	1.56	2.5	1.11	1.17			
2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.36	2.5		0.12	2.5
0.87	2.42	2.5	2.5	2.5	0.90	2.5	2.5	2.5		0.09	2.5
										neg control	pos

HIV control 2

day 16

-1	-2	-3	-4	-5	-6	-7	-8	-9			
nd	nd	nd	nd	nd	1.80	2.44	2.5	0.77			
					2.5	1.93	2.5	2.5			
					1.99	2.5	2.5	2.5			
					2.5	2.5	2.5	2.5			
					2.5	2.5	2.5	2.5			
					2.5	2.5	2.5	2.5			
					2.5	2.5	2.5	2.5		0.37	2.5
					2.5	2.5	2.5	2.5		0.36	2.5
										neg control	pos

day 21, HIV control 2

-1	-2	-3	-4	-5	-6	-7	-8	-9			
nd	nd	nd	nd	nd	1.86	0.58	2.5	0.73			
					2.5	0.97	2.5	2.5			
					1.07	2.5	2.5	2.5			
					2.5	2.5	2.5	2.5			
					2.5	2.5	2.5	2.5			
					2.5	2.5	2.5	2.5			
					2.5	2.5	2.5	2.5		0.43	2.5
					2.5	2.5	2.5	2.5		0.77	2.5
										neg control	pos

Heat treatment of HIV in bone

day 1, 10 min HIV inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9			
2.5	2.5	1.80	0.87	0.73	0.35	0.22	0.26	0.33			
2.5	2.5	1.04	1.21	0.36	0.38	0.19	0.18	0.56			
2.5	2.5	0.38	1.03	0.36	0.24	0.20	0.46	0.37			
2.5	2.5	0.56	0.52	0.49	0.29	0.16	0.34	0.32			
2.5	2.5	0.62	0.52	0.40	0.22	0.17	0.16	0.39			
2.5	2.5	0.52	0.27	0.19	0.17	0.15	0.22	0.32			
2.5	1.98	0.38	0.21	0.21	0.19	0.13	0.32	0.33		0.27	2.5
2.5	2.5	0.48	0.22	0.20	0.10	0.13	0.11	0.28		0.31	2.5
										neg control	pos

day 8, 10 min HIV inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9			
2.5	2.5	2.5	2.5	2.5	2.5	0.43	1.04	2.22			
2.5	2.5	2.5	2.5	2.5	2.5	1.98	2.5	0.75			
2.5	0.29	0.21	0.21	0.21	0.27	0.48	0.15	0.38			
2.5	2.5	2.5	2.5	2.5	0.96	0.30	0.21	0.31			
2.5	2.5	2.5	2.5	2.5	2.5	0.36	0.28	0.31			
2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.24	0.34			
2.5	2.5	2.5	2.5	2.5	2.5	1.93	0.25	0.23		0.30	2.5
2.5	2.5	2.5	2.5	2.5	2.5	2.04	2.5	0.73		0.29	2.5
										neg control	pos

day 16, 10 min HIV inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9			
2.5	2.5	2.5	1.84	2.5	2.5	1.72	2.5	2.5			
2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5			
2.5	0.55*	0.53*	0.17*	0.17*	0.14*	0.25*	0.54	0.31			
2.5	2.5	2.5	2.5	2.5	2.5	0.42	1.97	0.24			
2.5	2.5	2.5	2.5	2.5	2.5	0.56	1.08	0.60			
2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.19	0.43			
2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.16		0.20	2.5
2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5		0.23	2.5
										neg control	pos

* corresponds to the observation of lacking giant cell formation in this line

day 21, 10 min HIV inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9			
nd	nd	nd	nd	nd	2.5	2.5	2.5	2.5			
					2.5	2.5	2.5	2.5			
					0.95	1.01	0.41	0.37			
					2.5	0.67	2.5	0.43			
					2.5	1.42	2.29	0.32			
					2.5	2.5	0.26	0.64			
					2.5	2.5	2.5	0.37		0.33	2.5
					2.5	2.5	2.5	2.5		0.16	2.5
										neg control	pos

Heat treatment of HIV in bone

day 1, 30 min HIV inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9			
1.08	0.54	0.20	0.16	0.13	0.18	0.27	0.30	0.24			
0.97	0.39	0.15	0.12	0.13	0.13	0.20	0.29	0.31			
1.43	0.41	0.18	0.14	0.14	0.15	0.24	0.22	0.25			
0.83	0.33	0.13	0.16	0.17	0.17	0.27	0.23	0.24			
0.78	0.29	0.14	0.11	0.15	0.14	0.24	0.23	0.22			
0.70	0.26	0.13	0.12	0.19	0.12	0.23	0.24	0.27			
0.73	0.26	0.12	0.11	0.11	0.14	0.16	0.19	0.20		0.25	2.5
0.75	0.27	0.18	0.12	0.12	0.22	0.33	0.17	0.31		0.29	2.5
										neg control	pos

day 8, 30 min HIV inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9			
1.15	0.51	0.23	nd	nd	nd	nd	nd	nd			
1.22	0.51	0.19									
1.74	0.53	0.22									
1.09	0.38	0.17									
0.69	0.31	0.18									
0.85	0.31	0.20									
0.82	0.31	0.19								0.24	2.5
1.00	0.36	0.22								0.23	2.5
										neg control	pos

day 16, 30 min HIV inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9			
2.5#	0.99	0.34	nd	nd	nd	nd	nd	nd			
1.84	1.01	0.45									
2.5	1.25	0.41									
1.70	0.12	0.38									
1.43	1.16	0.44									
1.33	0.86	0.78									
1.30	0.52	0.34								0.20	2.5
1.99	0.78	0.31								0.61	2.5
										neg control	pos

day 21, HIV 30 min inactivation

-1	-2	-3	-4	-5	-6	-7	-8	-9			
2.16#	0.76	0.29									
1.33	0.64	0.36									
1.33	0.61	0.30									
1.27	0.46	0.36									
1.10	0.38	0.44									
0.73	0.33	0.43									
0.87	0.27	0.32								0.54	2.5
1.29	0.32	0.30								0.48	2.5
										neg control	pos

this well showed giant cell formation,not the other wells of the same row

HIV heat treatment in bone

day 1, 50 min HIV inactivation

-1	-2	-3	-4	-5	-6						
0.52	0.56	0.28	0.30	0.20	0.24						
0.64	0.35	0.42	0.43	0.41	0.20						
0.62	0.35	0.20	0.33	0.25	0.25						
0.54	0.37	0.20	019	0.27	0.20						
0.48	0.35	0.22	0.36	0.37	0.16						
0.40	0.26	0.26	0.16	0.18	0.23						
0.40	0.31	0.12	0.17	0.16	0.15					0.22	2.5
0.41	0.19	0.13	0.11	0.12	0.14					0.29	2.5
										neg control	pos

day 8, 50 min HIV inactivation

-1	-2	-3	-4	-5	-6						
0.91	0.70	0.25	nd	nd	nd						
0.77	0.50	0.25									
0.68	0.59	0.25									
0.56	0.52	0.27									
0.61	0.60	0.27									
0.66	0.38	0.38									
0.54	0.41	0.25								0.33	2.5
0.70	0.27	0.19								0.33	2.5
										neg control	pos

day 16, 50 min HIV inactivation

-1	-2	-3	-4	-5	-6						
0.84	0.62	nd	nd	nd	nd						
0.78	0.40										
0.57	0.49										
0.63	0.50										
0.47	0.28										
0.47	0.27										
0.40	0.29									0.17	2.5
0.53	0.25									0.14	2.5
										neg control	pos

day 21, 50 min HIV inactivation

-1	-2	-3	-4	-5	-6						
1.17	0.57	nd	nd	nd	nd						
0.82	0.47										
0.85	0.52										
0.53	0.27										
0.51	0.32										
0.42	0.25										
0.42	0.40									0.18	2.5
0.52	0.22									0.25	2.5
										neg control	pos

Addendum to the evaluation of heat inactivation of HIV in human femur heads

Statistical information:
To the log calculation of TCID50 given in the first report Poisson distribution and 95% confidence intervals are included. Calculations have been made with the Stat Mate Program (1) from Graph Pad Software, San Diego, California.
1. Motulsky H. Intuitive Biostatistics, Oxford University Press, 1995

Statistical ranges given are from day 21 of culture, that means that last day when the titer had reached end point. Cum probability is cumulative probability.

Experiment 1

time of heat inactivation	TCID titer log 10	Poisson distribution probability	cum probability	95% confidence interval
control 1	10.12	0.1126	0.3328	0.8942 - 0.9922
5 min	8.18	0.0631	0.130	0.6267 - 0.8299
20 min	5.38	0.0378	0.067	0.484 - 0.7078
40 min	0	0	0	0.000 - 0.045*
62 min	0	0	0	0.000 - 0.045*

* this is a 97.5% confidence interval

Experiment 2

time of heat inactivation	TCID titer log 10	Poisson distribution probability	cum probability	95% confidence interval
control 2	10.38	0.0631	0.1301	0.6540 - 0.8507
10 min	7.44	0.0378	0.0670	0.5230 - 0.7422
30 min	1.18	0.0005	0.005	0.0206 - 0.1400
50 min	0	0	0	0.000 - 0.045*

* this is a 97.5% confidence interval

Addendum 2 to the evaluation of heat inactivation of HIV in human femoral heads

This addendum describes the statistical limitations of the inactivation performed according to the log dilution and small volume (100 µL) used for the spiking of the femoral head.

1. **Calculation** of the limit of detection of HIV by the procedure used

The formula is based on the proposal made by Prigge et al (6) and described in the "Bekanntmachung über die Zulassung von Arzneimitteln" of the Paul-Ehrlich-Institut (7):
$p = [(V-v)/V]^n$
p is generally accepted with 0.05
V was 100 µL and v was 80 µL
n means the number of undetected virus in the assay.

$0.05 = [(100-80)/100]^n$, which is n = 1.86 since in all 8 wells no HIV was finally detectable

Therefore the limit of detection is 1.86 per 100 µL, which is 18.6 per mL, which is $10^{1.27}$.
This limit of 10 log 1.27 corresponds to the line in the following Figure. Figure 4 is equivalent to Figure 3 of page 6A of the evaluation of Nov.1995.

2. **Graph** of the destruction of HIV d ..ing heating femur heads by heat with the limit of virus detection

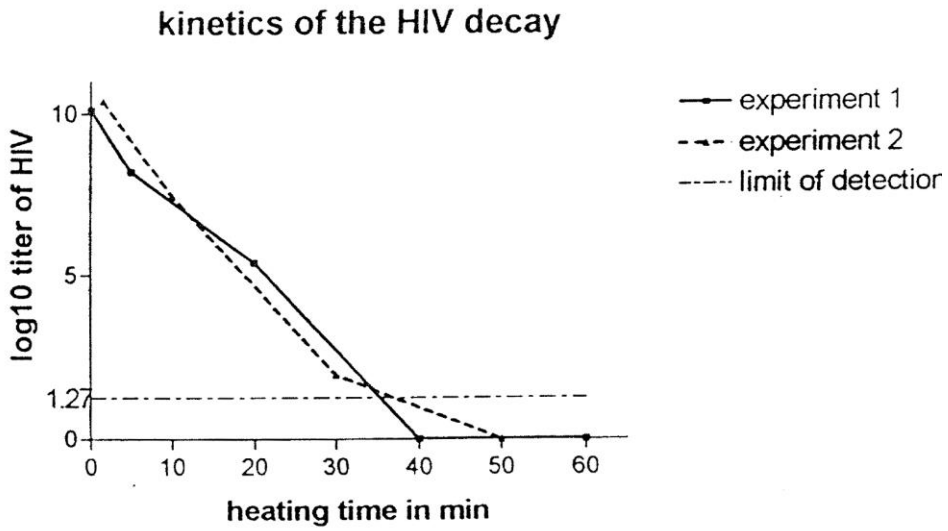

Figure 4: Kinetics of the HIV decay by heat treatment to 80°C and indication of the limits of detection of the procedure used for titrating HIV.

6. Prigge R, Günther O, Bonin O, Eissner G, Hallervorden J, Spaar JW. Probleme der staatlichen Prüfung von Poliomyelitis Impfstoffen. Dtsch Med. Wschr. 1956; 81: 1-9
7. Bundesgesundheitsamt und Paul Ehrlich Institut. Bekanntmachung über die Zulassung von Arzneimitteln. Anforderungen an Validierungsstudien zum Nachweis der Virussicherheit von Arzneimitteln aus menschlichem Blut oder Plasma. Bundesanzeiger Mai 1994; 84
München, 20. Mai 1996

Prof. J. Gürtler

Addendum 3 to the evaluation of heat inactivation of HIV in femur heads - standby control

This addendum has been performed to measure the spontaneous inactivation of HIV in a separate tube on the laboratory bench at room temperature during the 62 min period, in which other tubes had been positioned in the femoral heads and exposed to heat. Experimental conditions were as described previously on page 2 and 3 under Materials and Methods.

Results

In this experiment a final HIV titer after 3 weeks of culture on HUT-78 cells of 10.25 log 10 was obtained which was at starting time 1.31 and after one week of incubation 10.44. When the titration titers were measured after keeping the tubes for 62 min at the lab bench the starting titer was 2.0 log 10 , after one week 9.0 and after the second week, which was the last measurement 9.63. A two weeks observation period was chosen, since in the previous experiments between 2 and 3 weeks there was only a marginal difference in the HIV titer (Figure 2 page 6).

The decline of the HIV titer is shown in Figure 5.

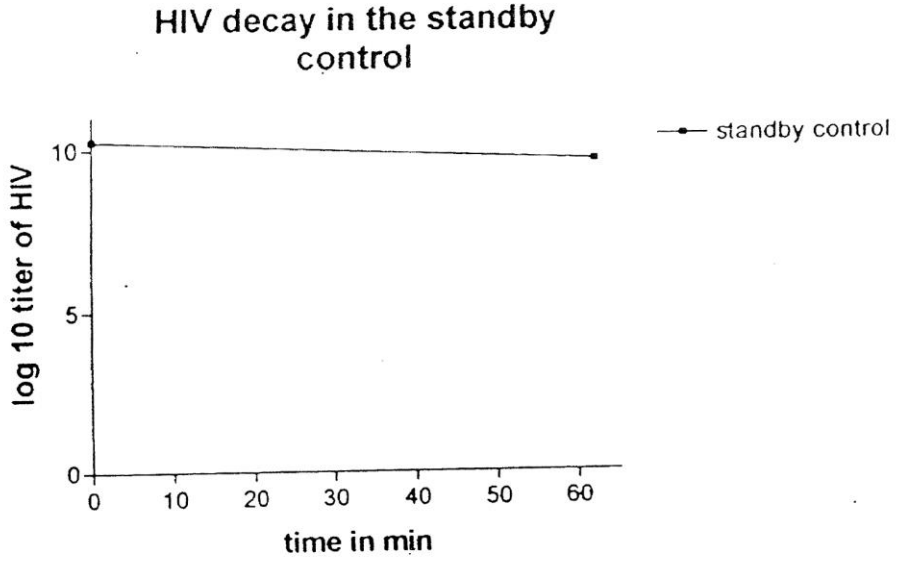

Figure 5. Decline of the HIV titer after keeping the HIV solution with HUT-78 cells for 62 min at room temperature. A slight decline from 10.25 to 9.63 log 10 was observed.

Statistical evaluation

To get further information on the significane of the range of the decline of the HIV titer found the Poisson distribution and the 95% confidence interval were calculated, according to Motulski (see reference 1 on page 27). The obtained data are shown in the following table.

time of keeping at room temperature	TCID titer log 10	Poisson distribution probability	cumul.probability	95% confidence interval
0 min	10.25	0.113	0.333	0.444 - 0.975
62 min	9.63	0.132	0.587	0.517 - 0.997

Discussion

The HIV titer obtained in this independent titration was very close to the titers of the previous experiments, which were 10.12 and 10.38 log 10. The range of variation is narrow in all 3 experiments, but when calculated according to the 95% confidence interval, is equal. The decline found after 1 hour incubation is lower than at starting time, but is still within the range of variation. Compared to the 5 min titer in the previous experiment which was 8.18 log 10, the 62 min value is within the trend of showing a decline, but does not demand a recalculation of the rate of HIV decay described.

München, 20. Juli 1996

Prof. J. Gürtler

Extincions of the p24-antigen assay in the wells of the microtiter plates of the standby control 0 min

day 1

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
2.5	0.863	0.256	0.228	0.137	0.162	0.091	0.127	0.085	0.109		
0.126	0.457	0.160	0.131	0.160	0.166	0.176	0.218	0.149	0.130		
2.5	0.923	0.266	0.169	0.119	0.187	0.173	0.167	0.147	0.171		
2.5	1.228	0.293	0.249	0.159	0.298	0.151	0.186	0.163	0.178		
2.5	1.185	0.517	0.188	0.217	0.233	0.136	0.128	0.118	0.147		
2.5	1.144	0.272	0.412	0.203	0.236	0.234	0.419	0.131	0.143		
2.5	0.896	0.319	0.256	0.135	0.226	0.304	0.200	0.235	0.194		2.5
2.5	0.114	0.303	0.182	0.128	0.120	0.151	0.127	0.102	0.158	0.145	2.5
										neg control	pos

day 7

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
2.5	2.5	2.5	1.858	2.475	0.192	0.211	0.162	0.121	0.086		
0.073	0.167	0.108	.312	.258	0.263	0.241	0.418	0.113	0.113		
2.5	2.5	2.5	2.5	2.5	0.392	0.275	0.328	0.146	0.159		
2.5	2.5	2.5	2.5	2.052	1.590	0.232	0.339	0.137	0.106		
2.5	2.5	2.5	2.5	2.234	0.654	0.193	0.188	0.131	0.159		
2.5	2.5	2.5	2.5	2.5	0.690	0.199	0.161	0.112	2.101		
2.5	2.5	2.5	2.5	1.549	0.342	0.388	0.225	0.392	2.5		2.5
2.5	2.5	2.5	2.5	2.5	0.517	0.258	0.175	0.165	2.5	0.150	2.5
										neg control	pos

day 14

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
nd	nd	nd	nd	2.5	2.5	2.5	0.129	0.095	0.137		
				0.133	0.209	0.259	0.225	0.272	0.192		
				2.5	2.5	0.252	0.268	0.182	0.172		
				2.5	2.5	2.5	2.5	0.195	0.315		
				2.5	2.5	2.5	0.319	0.190	0.267		
				2.5	2.5	0.195	0.234	0.397	2.5		
				2.5	2.5	2.5	2.5	2.5	2.5		2.5
				2.5	2.5	2.5	0.137	2.5	2.5	0.122	2.5
										neg	pos

Extinctions of different wells in the p24-antigen assay in the 62 min standby control

day 1

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
2.5	1.380	0.683	0.144	0.207	0.101	0.397	0.121	0.110	0.072		
2.5	1.145	0.405	0.295	0.418	0.563	0.235	0.277	0.164	0.242		
2.5	1.643	0.662	0.281	0.211	0.301	0.361	0.241	0.251	0.132		
2.5	1.408	0.635	0.382	0.251	0.405	0.288	0.337	0.133	0.313		
2.5	1.763	0.844	0.336	0.278	0.304	0.380	0.359	0.206	0.289		
2.5	1.415	0.523	0.313	0.336	0.260	0.243	0.358	0.193	0.277		
2.5	1.597	0.520	0.303	0.304	0.443	0.424	0.280	0.280	0.305	0.221	2.5
2.5	2.5	1.105	0.351	0.209	0.343	0.320	0.448	0.265	0.218	0.151	2.5
										neg control	pos

day 7

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
2.5	2.5	2.5	2.5	2.5	2.5	0.124	0.146	0.359	0.141		
2.5	2.5	2.5	2.5	2.5	2.5	0.840	0.465	0.878	0.338		
2.5	2.5	2.5	2.5	2.5	1.211	1.264	0.409	1.983	0.252		
2.5	2.5	2.5	2.5	2.5	2.273	0.674	0.299	0.751	0.336		
2.5	2.5	2.5	2.5	2.5	1.955	0.813	0.459	1.400	0.809		
2.5	2.5	2.5	2.5	2.5	2.089	0.596	0.293	1.155	0.733		
2.5	2.5	2.5	2.5	2.5	2.064	0.731	0.987	1.058	0.704	0.217	2.5
2.5	2.5	2.5	2.5	2.5	2.5	2.5	1.520	2.5	0.313	0.214	2.5
										neg control	pos

day 14

-1	-2	-3	-4	-5	-6	-7	-8	-9	-10		
nd	nd	nd	nd	nd	nd	0.90	0.210	2.5	0.093		
						2.5	2.5	2.5	2.5		
						2.5	2.5	2.5	0.110		
						2.5	2.5	0.168	2.5		
						2.5	2.5	2.5	2.5		
						2.5	0.192	0.096	0.176		
						2.5	0.884	2.5	2.5	0.219	2.5
						2.5	2.5	2.5	2.5	0.250	2.5
										neg control	pos