Early kinetics of innate and adaptive immune responses during hepatitis B virus infection

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ABSTRACT

Background and Aim: Innate immunity appears to be silent in acutely hepatitis B virus (HBV)-infected chimpanzees, as shown by microarray analysis of intrahepatic gene expression. Whether this observation also applies to HBV pathogenesis in man remains undefined. The aim of this study was thus to characterise natural killer (NK) and CD56+ natural T (NT) cell responses early after human HBV infection and their relationship to the induction of adaptive immunity.

Methods: Two HBV-seronegative blood donors who became hepatitis B surface antigen (HBsAg) and HBV DNA positive but had persistently normal alanine aminotransferase (ALT) were followed from a very early stage of HBV infection. The phenotype (CD69 and NKG2D) and function (cytokotoxicity and interferon γ (IFNγ) production) of NK and NT cells were analysed. CD4- and CD8-mediated responses were studied in parallel with overlapping peptides covering the entire HBV sequence by ex vivo intracellular cytokine staining (ICS) for IFNγ, interleukin 2 (IL2), IL4 and IL10, and by ex vivo Elispot for IFNγ, Healthy subjects, and patients with chronic and acute HBV infection were studied for comparison.

Results: An early induction of both innate and adaptive responses was observed. NK and NT cells showed faster kinetics than HBV-specific T cells with an earlier peak of activity, while CD4+ and CD8+ cell responses were mounted with a similar profile, with higher frequencies of IFNγ-producing CD8+ cells at the peak of the response.

Conclusions: The innate immune system is able to sense HBV infection, as shown by the early development of NK and NT cell responses, which probably contribute to contain the HBV infection and to allow timely induction of adaptive responses.

Patients and methods

This study was conducted on two previously seronegative blood donors who were found to have seroconverted with the appearance of HBsAg during their periodic serological and virological screening which was conducted every 3 months. Both patients were male, 32 and 46 years old, respectively. While for patient 1 no information was available about the route of transmission, patient 2 probably acquired HBV infection by the sexual route approximately 1 month before HBsAg was first detected. They had not been previously vaccinated against HBV and did not undergo antiviral treatment during HBV infection.

In addition, eight patients with clinical, biochemical and virological evidence of acute HBV infection (aminotransferase levels at least 10 times the upper limit of the normal range, positive HBsAg and immunoglobulin M (IgM) anti-hepatitis B core (Hbc) antibodies in the serum) were studied; also two patients with chronic hepatitis B and three healthy uninfected subjects were analysed as controls. All patients were negative for anti-hepatitis C virus, delta...
virus, HIV-1 and HIV-2 antibodies, and for serum markers of autoimmune hepatitis.

HBsAg, anti-HBs, total and IgM anti-HBc, hepatitis B envelope (HBe), anti-HBe, anti-hepatitis D virus, anti-hepatitis C virus, and anti-HIV-1 and -2 were tested by commercial enzyme immunoassay kits (Abbott Labs, Abbott Park, Illinois, USA; Ortho-Clinical Diagnostic, Raritan, New Jersey, USA; Johnson & Johnson, New Brunswick, New Jersey, USA; DiaSorin, Vercelli, Italy). Quantitative HBV DNA was analysed by PCR (Cobas Amplicor test; Roche Diagnostics, Basel, Switzerland). The study was approved by the ethical committee of the Azienda Ospedaliero-Universitaria of Parma, Italy, and all subjects gave written informed consent to participate in the study.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinised blood by Ficoll-Hypaque density gradient centrifugation, and cryopreserved. Samples from all different time points were thawed simultaneously to perform assays under the same experimental conditions and avoid interassay variability. After thawing, PBMCs were resuspended in RPMI 1640 supplemented with 25 mmol/l HEPES, 2 mmol/l L-glutamine, 50 µg/ml gentamycin and 8% human serum (complete medium) for immunological analysis.

Phenotypic studies

For direct NK and CD56+ natural T (NT) cell phenotypic studies, 5×10^6 PBMCs were incubated for 15 min in the dark at room temperature with anti-CD56-fluorescein isothiocyanate (FITC) (BD Biosciences-Pharmingen, San Jose, California, USA), anti-CD3–peridinin chlorophyll protein (PerCP) (BD Biosciences-Pharmingen) and anti-NKG2D–phycocerythrin (PE) (R&D Systems, Minneapolis, Minnesota, USA) or anti-CD69–PE (BD Biosciences-Pharmingen) antibodies. Cells were then washed with phosphate-buffered saline (PBS)–0.1% fetal calf serum (FCS) and analysed immediately on a BD Bioscienes flow cytometer (FACSCalibur) using CellQuest software.

Interferon γ (IFNγ) production by NK and NT cells

To determine the capacity of NK and NT cells to produce IFNγ, 1×10^6 PBMCs were incubated for 18 h at 37°C with or without 1.25 µg/ml interleukin 12 (IL12; Sigma-Aldrich, St Louis, Missouri, USA); brefeldin A (Sigma) (10 µg/ml) was then added for the last 5 h of incubation, before surface staining with anti-CD56 and anti-CD3, as described above. Cells were fixed for 15 min at room temperature with 50 µl of Fix and Perm medium A (Caltag Laboratories, Burlingame, California, USA), washed once, permeabilised with 50 µl of medium B (Caltag) and stained with PE-conjugated anti-IFNγ (Sigma) for 15 min at room temperature. After a washing step, cells were then analysed by flow cytometry. The percentage of cytokine releasing was evaluated by subtracting the values obtained by unstimulated samples from those of IL12-stimulated samples.

NK cell-mediated cytotoxicity assay

NK cell-mediated cytotoxic activity was tested in a standard 4 h 51Cr-release assay, where K562 cells were used as target cells at different effector:target (E:T) ratios ranging from 40:1 to 5:1, following overnight incubation of PBMCs with or without 1 ng/ml of IL15 (Sigma). A total of 1×10^6 target cells were incubated with 100 µCi of Na251CrO4 at 37°C for 1 h, washed to remove free radioactivity and resuspended in complete medium. Triplicate cultures of 5×10^6 target cells were incubated with PBMCs at twofold decremental E:T ratios ranging from 40:1 to 5:1. Plates were then incubated at 37°C for 4 h. After incubation, 25 µl aliquots of supernatant were harvested, and the released radioactivity was determined in a gamma counter. The percentage lysis was calculated as:100×((experimental–spontaneous release)/total–spontaneous release). Percentage lysis values were normalised to NK/total PBMC percentages, as obtained by flow cytometry analysis (killing efficiency).

Ex vivo IFNγ Elispot assay

IFNγ Elispot assay was performed using a panel of 315 overlapping 15-mer peptides spanning the sequence of all HBV proteins, and pooled in 16 mixtures as already described.5 Multiscreen-IP 96-well plates (Millipore, Malshelm, France) were coated overnight at 4°C, as recommended by the manufacturer, with 5 µg/ml capture mouse anti-human IFNγ monoclonal antibody (IDIK; Mabtech, Sweden). Plates were then washed seven times with PBS–0.05% Tween 20, and blocked with RPMI 1640–10% FCS for 2 h at 37°C. PBMCs (2×10^6/well) were seeded in duplicate for each individual peptide mixture. Plates were incubated for 18 h at 37°C in the presence or absence of peptides. After washing, 50 µl of 1 µg/ml biotinylated secondary mouse anti-human IFNγ monoclonal antibody (786-1; Mabtech, Sweden) was added. After 5 h of incubation at room temperature, plates were washed four times; 100 µl of goat alkaline phosphatase antibinin antibody (Vector Laboratories, Burlingame, California, USA) was added to the wells, and the plates were incubated for a further 2 h at room temperature. Plates were then washed four times, and 75 µl of alkaline phosphatase-conjugated substrate (5-bromo-4-chloro-3-indolyl phosphate; Bio-Rad Laboratories, Hercules, California) was added. After 4–7 min., the colorimetric reaction was stopped by washing with distilled water. Plates were air dried, and spots were counted using an automated ELISPOT reader (AID ELISPOT reader system; Autoimmune Diagnostika, Strassberg, Germany). Results are expressed as numbers of spot-forming units (SFU) per 1×10^6 PBMCs. The number of specific IFNγ-secreting cells was calculated by subtracting the value of the unstimulated control from the value of the stimulated sample. The positive control consisted of PBMCs stimulated with phytohaemagglutinin. Wells were considered positive if they had values of least twice the background value and the number of spots was >10.

Intracellular cytokine staining

A total of 1×10^6 PBMCs were stained to test IFNγ, IL2, IL10 and IL4 production. Cytokine production was assessed by using 16 pools of 15-mer overlapping peptides covering the entire HBV sequence. PBMCs were marked as already described: anti-CD4–PE or anti-CD8–PE and anti-CD3–PerCP (BD Pharmingen) were used for surface staining; anti-IFNγ–FITC or IL4–FITC (Sigma), and anti-IL2–APC (allophycocyanin) or anti-IL10–APC antibodies (BD Pharmingen) were used for intracellular cytokine staining.

RESULTS

The evolution of HBV infection in two infected blood donors is illustrated in fig 1. In patient 1, at the time HBsAg was first detected in the serum, HBeAg and anti-HBc IgM were still negative; HBV viraemia was barely detectable and viraemia levels remained stably low for 3–4 weeks, thereafter started a rapid rise to 2–3×10^6 copies/ml, a level which was maintained for approximately 3 weeks, and then dropped rapidly to...
Figure 1  Phenotypic and functional characterization of natural killer (NK) and CD56+ natural T (NT) cell-mediated responses. At the top, serological, biochemical and virological features of the two patients with acute hepatitis B virus (HBV) infection and persistently normal alanine aminotransferase (ALT) levels are shown. HBV markers, ALT levels and serum HBV DNA concentration were analysed longitudinally in the two blood donors at the time points illustrated on the horizontal axis, as weeks from the first hepatitis B surface antigen (HBsAg) detection. Before detection of HBsAg, they always tested negative for HBsAg and HBV DNA. (A) Longitudinal analysis of CD69 and NKG2D expression on NK (CD56+CD3–) and NT (CD56+CD3+) cells. NKG2D expression is illustrated as mean fluorescence intensity (MFI). (B) NK cytotoxic activity and interferon γ (IFNγ) production by NK and NT cells.
undetectable levels (fig 1). The follow-up of the second patient started apparently later after infection because not only HBsAg but also HBeAg were already positive in the first available serum sample; moreover, HBV DNA levels were closer to their maximal peak which was reached a week later (fig 1). For both patients, HBsAg was negative at a previous evaluation performed approximately 3 months earlier, when only serum was available for analysis; serum ALT remained completely normal during the overall follow-up, since transaminase levels never exceeded 40 U/L. To investigate the temporal relationship between induction of innate and adaptive cellular immunity, evolution of HBV-specific T cell responses was analysed in parallel to NK cell activity and to the function of CD56\(^+\)CD56\(^-\) cells. These are not classical invariant NKT cells, but are a broader population of NK receptor-positive T cells with dual innate and adaptive immune functions. Early diagnosis of infection allowed us also to ask whether the kinetics of CD4 and CD8 expansion following antigen encounter differ in vivo, and whether the frequency of virus-specific CD8 and CD4 IFN-\(\gamma\)- or IL2-producing cells is different at the peak of the T cell response. In addition, we asked which was the sequence of HBV antigens targeted by T cells for a successful development of the protective cell-mediated immune response.

**Evolution of phenotype and function of NK and NT cells after infection**

Consistent with the important role of innate immunity during the early phase of viral infections, high expression of activation markers, such as CD69 and NKG2D, on the surface of CD56\(^-\)CD56\(^-\) NK and CD56\(^-\)CD56\(^-\) NT cells was already detectable at the time of the first determination, consistent with a very early activation of these cells in the course of HBV infection (fig 1A). To study NK cell function, cytotoxic activity and IFN-\(\gamma\) secretion were then analysed. NK cell-mediated cytotoxic activity was tested in a standard 4 h \(^{51}\)Cr-release assay, where K562 cells were used as targets at different E:T ratios ranging from 40:1 to 5:1. In patient 1, NK cytotoxic activity increased progressively, reaching maximal levels corresponding with the viraemia peak and decreasing thereafter (fig 1B). In patient 2, who was presumably followed from a more advanced time point after infection, NK cytotoxic activity appeared to be slightly diminishing over time from the initial time points, suggesting that it probably had already reached its peak (fig 1B). A behaviour similar to cytotoxicity was also observed for IFN-\(\gamma\) production by NK and NT cells since NK cells reached a peak concurrently with the peak of viraemia, while CD8\(^+\)CD56\(^-\) NT cells reached maximal IFN-\(\gamma\) production 2–3 weeks before NK cells (figs 1B and 2A).

Control longitudinal analysis of NK cell activation markers and NK cell cytotoxic activity in healthy subjects and patients with typical acute or chronic HBV infection showed that both phenotypic and functional parameters remained stable over time without fluctuations comparable with those detected in patient 1; moreover, their levels were generally lower in the control groups than in patient 1 (fig 5). Thus, the kinetics of NK cell responses should actually be related to acute virus infection rather than expression of physiological intrairandinal fluctuations.

**Early kinetics of ex vivo CD4 and CD8 T cell responses**

To study the evolution of adaptive immune responses in relation to NK and NT cell activity, ex vivo cytokine production by circulating HBV-specific T cells was first analysed by intracellular cytokine staining (ICS) for IFN-\(\gamma\), IL2, IL4 and IL10, using a panel of overlapping 15-mer peptides spanning the sequence of all HBV proteins, and pooled in 16 peptide mixtures, as previously described. At very early time points, T cell responses were already detectable, but still very weak (fig 4A), confirming previous tetramer staining data. The intensity of T cell responses increased progressively, reaching maximal IFN-\(\gamma\) production in both subjects at a time when viraemia was already undetectable, and diminishing thereafter (figs 4A and 2B). At the time of the maximal peak, IFN-\(\gamma\) production was sustained, mainly for patient 1 and completely for patient 2, by the CD8\(^+\) T cell subset (figs 4A and 2B), supporting a crucial role for CD8\(^+\) cells in viral control, in accordance with previous studies. IL2 secretion by CD4\(^+\) and CD8\(^+\) T cells reached a peak before IFN-\(\gamma\), while IL4 and IL10 production by CD4 cells progressively increased from the early time points, and maximal production was observed when secretion of Th1 cytokines was already declining and the anti-HBs antibody titre was increasing in the serum (fig 4A). The level of T cell reactivity was similar to what is frequently detectable in typical acute HBV patients, as illustrated for comparison in fig 5, where eight patients with acute HBV infections are shown. Thus, by ex vivo longitudinal analysis of NK-, NT-, CD8- and CD4-mediated responses, the peak of NK and NT cell activity was detected when HBV DNA was maximal. At this stage CD8 and CD4 responses were still very weak, reaching their maximal activity when HBV viraemia and NK/NT responses were rapidly declining (fig 4B).

**Sequential role of HBV proteins in T cell activation**

Ex vivo T cell responses to individual peptide mixtures were also analysed by IFN-\(\gamma\) Elispot (fig 4C). In patient 1, peptides of the envelope and polymerase regions were initially targeted by T cells at the first time point analysed (145 and 45 spot-forming units (SFU)/10\(^6\) PBMCs, respectively). The results were confirmed by means of stimulation with the same peptide mixtures used for ex vivo analysis, showing that early responses were predominantly induced by envelope and polymerase peptides (data not shown). Responses to HBV core peptides were mounted later, immediately before HBV DNA became undetectable (662 SFU/10\(^6\) PBMCs). In patient 2, T cell responses to HBV envelope peptides (50 SFU/10\(^6\) PBMCs) were the first to be detected when HBV DNA was still elevated (fig 4C); reactivity to HBV core peptides emerged when viraemia dropped (130 SFU/10\(^6\) PBMCs); finally, responses to HBV polymerase peptides (85 SFU/10\(^6\) PBMCs) were the last to appear. Therefore, in both patients, the earliest IFN-\(\gamma\) Elispot responses were stimulated by the HBeAg while detection of the most vigorous responses, which were directed against core peptides, coincided with viral decline. By intracellular cytokine staining with the individual peptides contained in the HBC peptide mixtures, the response was shown to be focused on two peptides: the first one...
comprising amino acids 81–95 of the core protein elicited IFN-γ production only by CD8+ cells (0.11% of total CD8+ T cells); and the second one spanning amino acids 131–145 induced IFN-γ (0.22% of CD8+ cells) and IL2 (0.06% of CD8+ cells) production not only by CD8+ cells but also by CD4+ cells (IFN-γ, 0.05% of CD4+ cells; IL2, 0.03% of CD4+ cells) (data not shown).

**DISCUSSION**

HBV infections acquired in adult life are generally self-limiting, and recovery from infection is associated with the development of efficient adaptive immune responses which allow sustained control of infection with maturation of long-lasting T cell memory. Priming of protective T cell responses has been shown in different models of virus infection to be greatly affected by the cross-talk between NK and dendritic cells (DCs), where NK cells play a regulatory function essential for DC maturation and subsequent T cell priming. Thus, in the course of a virus infection efficiently controlled by the host immune system, as in HBV infection acquired in adult life, NK cell activity is expected to play a key role in skewing T cell priming and differentiation. Unexpectedly, however, none of the antiviral genes that reflect the activation of the innate immune system is induced or repressed by HBV in the liver of infected chimpanzees, implying that HBV infection does not activate an intrahepatic innate immune response. These results are totally different from those previously reported applying the same experimental approach to HCV infection where innate immune response-associated genes were identified, reflecting a...
timely activation of the innate immune system following HCV infection. The lack of early intrahepatic gene induction may be a peculiar feature of the chimpanzee model of HBV infection, because an early activation of NK and NKT cells occurring 48–72 h after infection has been reported in woodchuck infection with high doses of woodchuck hepatitis virus. Moreover, longitudinal analysis of circulating NK cells shows that their frequency is maximal in the incubation period of natural HBV infection with a subsequent decline at the time of the decrease in HBV DNA. No information about NK cell function early in natural human infection is, however, available because the incubation period of HBV infection is always asymptomatic and therefore difficult to study. Thus, whether HBV actually behaves as a "stealth" virus which also does not induce innate responses in natural HBV infection, as in chimpanzees, is still largely undefined. To address this issue and to characterise better the behaviour of two important cellular components of the innate immune system in the early phase of HBV infection, we analysed longitudinally the frequency and function of CD56^+CD3^- NK and CD56^+CD3^+ NT cells in two blood donors who were found to be HbsAg positive during the serological controls which are routinely performed before blood donation. Both were HbsAg negative at the previous control performed approximately 3 months before. In patient 1, HBeAg was still negative at the first control; in both patients, anti-HBc IgM was undetectable and serum HBV DNA was still rising. Evolution of HBV-specific T cell responses was analysed in parallel to define better the sequence of early immune events which are associated with successful control of infection, since the two patients were able to eliminate the virus.

Figure 3  Longitudinal analysis of natural killer (NK) cell phenotype and cytotoxicity in healthy uninfected subjects, and acute and chronic hepatitis B virus (HBV) patients. Time points are indicated as weeks from the first determination. For acute HBV patients, the first time point coincides with clinical presentation. E:T ratio, effector:target ratio; MFI, mean fluorescence intensity.
without detectable serum ALT elevation. Early diagnosis of infection also gave the opportunity to ask whether the kinetics of CD4+ and CD8+ cell expansion following antigen encounter differ in vivo, and whether the frequency of virus-specific IFNγ- or IL2-producing CD8+ and CD4+ cells is substantially different at the peak of the T cell response. After antigen exposure, CD8+ cells have been shown to have a faster rate of cell division, and the frequency of virus-specific CD8+ cells has been reported to be higher than that of CD4+ cells at the peak of the antiviral response in different mouse models of virus infection. 21–24

Figure 4 Ex vivo kinetics of the hepatitis B virus (HBV)-specific T cell response. (A) Ex vivo interferon γ (IFNγ) and interleukin 2 (IL2) production was tested on both CD8+ and CD4+ T cells, while IL10 and IL4 secretion was tested only on CD4+ T cells by intracellular cytokine staining, as described in the text, by using 16 pools of 15-mer peptides covering the entire HBV sequence. Each bar represents the total frequency of cytokine-positive cells among the overall CD4+ or CD8+ population at each individual time point, as determined by summing the responses to each individual peptide pool. (B) The different kinetics of IFNγ production by NK, CD8+ and CD4+ T cells during the course of HBV infection in the two subjects studied are illustrated for comparison. ND, not determined. (C) T cell responses to peptide pools corresponding to individual HBV proteins, as detected by IFNγ Elispot assay with 16 mixtures of 15-mer peptides, in relation to HBV viraemia. Each bar represents the sum of IFNγ-producing cells stimulated with the corresponding peptide pools, expressed as numbers of spot-forming units (SFU) per 1 x 10^6 peripheral blood mononuclear cells (PBMCs). The number of specific IFNγ-secreting cells was calculated by subtracting the value of the unstimulated control from the value of the stimulated sample.
Observations, however, are not univocal in all studies since higher pathogen-specific CD4+ cell frequencies have also been reported. In addition, we asked what were the kinetics of antiviral cytokines produced in a natural condition of successful control of infection and what was the sequence of HBV antigens targeted by T cells for the successful development of the protective cell-mediated immune response.

First, our data indicate that NK cells and NT cells are promptly activated before maximal HBV DNA elevation. This is indicated by the early increase in frequency of activated NK/NT cells expressing activation markers, such as CD69 and NKG2D. Similar kinetics were observed for NK and NT cell cytotoxicity and IFNγ production, confirming the early activation of these cell populations.

Secondly, HBV-specific T cell responses reached their peak later, when HBV DNA was already declining. This has previously been described and confirms that adaptive T cell responses mount rapidly following HBV DNA elevation. CD4+ and CD8-mediated responses showed a similar profile of expansion in the infected host since HBV-specific CD4+ and CD8+ cells reached their maximal frequency after a similar time interval from initial antigen exposure. However, the frequency of IFNγ-producing CD8+ cells was higher than that of IFNγ-producing CD4+ cells at the peak of response. Similar differences in magnitude of CD4+ and CD8-mediated T cell responses have been reported in mice infected with lymphocytic choriomeningitis virus (LCMV), Sendai or vaccinia viruses or with Listeria monocytogenes and in human Epstein–Barr virus (EBV) infection.

This difference in magnitude of CD4 and CD8 responses has been attributed to different factors including a greater intrinsic proliferative capacity of CD8+ cells, a better efficiency of antigen presentation to CD8+ cells, and a stronger promotion of early proliferative activity of CD8+ cells by IL2 and IL15. In our study, IL2 production by both CD4+ and CD8+ cells peaked before IFNγ, indicating that an early IL2 production is probably needed to allow the infected host to achieve virus control and antigen clearance in self-limited HBV infections. In both patients envelope peptides were targeted at early time points while responses to core peptides were maximal at the time of the decrease in HBV DNA. This is in keeping with previous proliferation studies with recombinant HBV proteins showing a temporal association between core-specific T cell responses and resolution of infection.

The patients studied did not show the typical disease profile of acute hepatitis B with sharp ALT elevation; therefore, the early and efficient induction of innate responses may have been able to drive control of infection by non-cytolytic mechanisms rather than by hepatocyte lysis through perforin/granzyme or TRAIL-mediated mechanisms, which have been reported to contribute to liver damage during hepatitis flares in chronic HBV infection. Although our study design does not allow us to distinguish between these possibilities, the results, however, do allow us to establish unequivocally that NK and CD56+CD3− NT cells are able to mount an early and efficient response to HBV and that the cellular component of the innate immune system is able to sense HBV from the beginning of infection.

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Competing interests: None.

Ethics approval: The study was approved by the ethical committee of the Azienda Ospedaliero-Universitaria of Parma, Italy.

Patient consent: All subjects gave written informed consent to participate in the study.

REFERENCES

Editor’s quiz: GI snapshot

Robin Spiller, editor

Crampy left upper quadrant pain in a 26-year-old male

CLINICAL PRESENTATION
A 26-year-old man presented with left upper quadrant abdominal pain for 1 week. The character of the pain was intermittent, cramping in nature, and especially aggravated after meals. No fever, chills, haematochezia or melena were reported. Physical examination revealed local tenderness over the left upper quadrant without rebound tenderness. The rest of the examination was unremarkable. The laboratory results showed normal blood cell counts and the biochemical studies were within normal limits. A plain abdomen film was obtained (fig 1).

QUESTION
What is the diagnosis?
See page 998 for the answer

This case is submitted by:

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Figure 1  Plain abdominal radiograph.