MIT GAMES TO TEACH

REPLICATE
On the Border of Life

Game Design Document

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Replicate:
On the Border of Life

Overview
The player controls the navigation of a virus as it travels through the human body via the circulatory system. While the player tries to find the target cells in the host organism that are slated for infection, the virion needs to evade the various defenses of the human immune system. When the player manages to find a viable target, the virus enters the cell and sheds its protein coat. Inside the cell, the virus has a limited amount of time to reproduce before the Cytotoxic Killer T Cells or other phagocytes destroy the cell. The player needs to make copies of the virus before phagocytosis occurs. Once replication is accomplished, the cell explodes, releasing copies of progeny virus back into the body.

The game will end, either when the human host dies from the symptoms of infection, or when the immune response of the host manages to disable all the viruses. The goal of the game is to keep the host alive while maintaining a certain level of viral reproduction and survival within the organs and vascular system of the host. Every half-minute, the game rewards the player with a transmission bonus based on the number of viruses alive in the body and the method of transmission of the virus. This game increases in pace and rewards the player for being able to survive for longer periods of artful game play, although the ultimate conclusion of each round of game play is the eventual extermination of the host or the virus.

Customization
Before beginning to play the game, the player can choose various characteristics for his or her virus or pick from a selection of viruses based on reality with pre-assigned attributes. Attributes include:

- Target cells - neurons; kidneys; epithelial surfaces; epidermis.
- Mutation capacity
- Baltimore classification
- Ability to alter surface glycoproteins, primarily in RNA viruses.
- Mimicry of epitopes present on host proteins. Eg. Measles, Cytomegalovirus
- Method of cell penetration
- Lysis or budding (release of progeny)
- Infection target (distributed or localized cells, moving cells)
- Maneuverability
- Rate of progeny synthesis
- Latency ability
- Mode of transmission
- Sensitivity to heat, acid, et cetera

Collections of attributes will affect a player’s point accumulation table according to the challenge that the attribute presents to the player. For instance, picking a virus that attacks cells of the immune system, such as HIV, will yield relatively low scores per Infection, as the procedure for reproducing also impairs the host’s defense against the
virus. Choosing a virus that mutates readily will also make the game much easier and thus yield a lower score for most activities. On the other hand, if the player chooses a virus with a very high rate of progeny synthesis, the game can reward the player with greater transmission bonuses for keeping the host alive over an extended play session.

**Migration**
Before infecting a target cell, the virion needs to travel around the body to find its target. There are four types of blood vessels, each with specific conditions for game play. These are the arteries, the veins, the capillaries and the heart.

The virus in its polyhedral protein coat, also known as a virion, begins any given level from a specific location in the body where that virus normally enters. This may be a wound, the blood vessels around the intestines, the lungs, or the eyes. Once in the circulation, the virion moves only slightly faster than the rate of the erythrocytes surrounding it, propelled by the current of the blood plasma pumped by the heart. The player has little control over the forward velocity of the virion.

The camera stays locked behind the virion as it moves through the blood vessels. The viewing plane is perpendicular to the axis passing through the center of the blood vessel. The player has control over the position of the virion in the x and y axes parallel to the viewing plane. Using the left thumb stick on a game controller, the player will spend much time maneuvering the virion to stay away from the sides of the blood vessel. If the virion bumps against the side of the blood vessel or collides with a non-threatening cell, it will ricochet and temporarily cause the player to lose control. The player may rotate the camera around the z-axis by using the left and right triggers (counterclockwise and clockwise, respectively). When passing through areas of inflammation, the player’s propulsion rate will increase, and he or she will need to avoid popping out through holes in vessel walls.

In the arteries, the virion moves as fast as any other cell in the blood stream and the player needs to steer the virion towards the correct area for Cellular Attack. The arteries branch to different parts of the body and the player needs to choose between several paths at each branch to reach the desired location. The player receives notification several seconds before reaching the branch in the form of a series of lines radiating from the center of the virion, indicating the directions in which the branches will separate. The lines shrink as the virion approaches the branch. When the lines touch the edge of the polyhedron, the virion is right next to the appropriate branch and will probably miss the turning if the player has not already begun to steer the virion in the appropriate direction. The lines indicating the correct route to that organ will appear in a different color from the other lines. The circle and the lines rotate according to the orientation of the camera; if the player uses the triggers to rotate the camera, the lines will rotate to reflect accurate and useful information back to the player.
If the player chooses the correct route to a target organ, the virion will enter the capillaries of that organ and the blood flow is slower. The organ features clumps of differently colored cells that are candidates for Cellular Attack. Pressing the ‘A’ button (for the Xbox controller) near a clump will automatically shift the player to Cellular Attack mode. If the player enters the incorrect organ, the capillaries merely resemble smaller arteries and the player will not be able to enter Cellular Attack mode. The virion needs to be careful not to bump into the vessel walls. Any bump causes the player to lose precious time regaining control of the virion. Clusters of neutrophils adhere to the inner endothelium of vessels close to the target cells causing a dangerous obstruction past which the player must first navigate prior to Cellular Attack.

Once through the capillaries, the virion enters veins that also have a slower flow rate. This is primarily a collection game, where the player needs to quickly steer the virion across the width of the veins to collect power-ups. The veins have segments divided by valves, so the player only has one possible power-up to collect at any time. At every heartbeat, the virion travels to the next segment in the vein, leaving behind uncollected power-ups for later. Collected power-ups regenerate after two complete circuits. Should the player fail to successfully enter Cellular Attack mode while adjacent to appropriate cells (perhaps due to high levels of Interferon or losing control of the virion) he or she will need to undertake another revolution around the body in an effort to search for other target cells.

The last valve (tricuspid) opens into the right ventricle of the heart, a magnificent, cavernous, tumultuous space with large valves opening and closing at a rate of 80 times per minute or more. The player must navigate through the pulmonary valve and into the pulmonary artery, at which point the player has an update/rest. The screen quickly reminds the player of the goals for this level. The player then re-enters the heart via the left atrium and mitral valve and must navigate through the left ventricle and aortic valve and out through the aorta and around the body once again. Then the player has another opportunity to find the correct cells. In general, it takes approximately 25 seconds to traverse the arteries, capillary bed and veins, barring collisions, power-ups and Cellular Attacks. Note that the player does not need to control the virion while it travels through the pulmonary circulation. This occurs automatically while the screen displays the goals for the level, and brings a full circuit to approximately 30 seconds. An experienced player may unlock a special mode for the virion to infect the lungs and travel through the pulmonary circulation in addition to the rest of the body.

Instead of a specific organ, the player might need to infect a specific type of cell in the bloodstream or in the vessel walls. As in the organs, the target cells will be lit in an easily identifiable color and may appear at any stage. These cells must be not be hard to come across; players should not have to spend much time waiting for the correct target to appear. To infect a cell, the virion needs to be adjacent to the cell and the player must press the ‘A’ button. Moving cells are more difficult to attack and should present players with higher scores for successful Infections.
It will be difficult to represent the relative scales of the objects in this game realistically. It will also be necessary to take liberties with the color palette of the game. Note that biology books, especially freshman texts, take similar liberties with scale and coloring. Only play testing can suggest appropriate balances of color and size exaggeration. Indicators, such as the branch-lines, can help orient the player if realistic scales are still playable and enjoyable.

**Power-ups**
There are several power-ups in the game. These appear as abstract icons in the blood circulation. When the virion moves over these power-ups, the player collects and lists them in the lower-right hand corner of the screen. A player may collect up to three power-ups at any one time; additional power-ups replace those currently held by a player.

To activate the third power-up collected thus far, the player needs to press the ‘B’ button. Pressing the trigger again will deactivate the power-up. After deactivating the power-up or after the power-up times-out, pressing ‘B’ will activate the next power-up in the queue.

**Localized performance reflective of the population**
In order to reflect the autonomous behavior of a large number of viruses in the human body, the performance of the player will not only affect the individual virion but also a percentage of all the viruses in the body. The player will, at all times, see a running count of the number of copies of itself currently in the host. Every collision with a threat reduces that count by a percentage, whereas every successful Infection increases that count proportionally. The player can see this progress by witnessing the appearance and increase in non-player-controlled virions travelling alongside the player’s virion. Similarly, the immune system eliminates a proportion of all the viruses in the host as the player makes mistakes.

**Interferon**
During an Infection, the infected cell releases Interferon. Interferon binds to the surface receptors of nearby uninfected cells. These uninfected cells begin to synthesize antiviral proteins that inhibit viral replication. The player is therefore more likely to successfully replicate inside cells that are some distance from other already infected cells. So, if the virus in question only attacks a very localized and particular group of cells, this becomes difficult. Hence, the uninfected neighbors of an infected cell become ‘safe’ from the advances of the player and immune to attack. A clump of target cells, thus, becomes ineligible for Infection once the player has attempted to attack one of the cells in the clump. If the player fails to execute a complete Infection of a cell, not only will the player lose control of the virus, the player can no longer attack that clump of cells. Even if Infection succeeds, the player cannot simply infect an adjacent cell after replication.

**Threats during Migration**
The virion will encounter several different cells in the blood vessels while attempting to locate targets for Cellular Attack. Other than the red blood cells and other non-threatening elements in the blood, there are also threats that will destroy the virus upon
contact. Collision with a non-threatening cell may also make the virion bounce into something more dangerous or miss a branching vessel.

Most threats destroy the player’s virion or render it inactive upon collision, essentially ‘killing’ the virion/player. The camera zooms back and the player takes control of the closest clone of the virion in the blood stream. If there are no virions left available in the blood stream, the game is over.

Some threats appear at the beginning of the game, such as B Lymphocytes, Monocytes, and Neutrophils. These increase in quantity as the player replicates. Collision into these threats make other threats appear, such as Antibodies and Killer T cells. Collision into a macrophage causes the T cells to be sensitized to the virion/antigen, thus stimulating T cell growth. Soon after, Killer T cells and Helper T cells will wreak havoc. If, by dexterity, a player manages to delay collision with a macrophage, then he can delay sensitization of T cells. Likewise, contact with a B Lymphocyte promotes B cell division, the proliferation of plasma cells, and the production of antibodies by plasma cells. Avoidance of contact with a B Lymphocyte can thus delay the inevitable appearance of antibodies.

Macrophages can extend pseudopods towards the virion if the player avoids colliding into a macrophage but passes too close. There are fixed macrophages and wandering macrophages, both of which are lethal and will engulf the virus. Meanwhile, Killer T cells and Antibodies are both deadly and will overpower all but the most skillful players in minutes or seconds.

Once antibodies appear in the game, most players will see a steady increase of antibodies. The sheer number of antibodies should quickly end the game after a certain number of collisions as virions fall prey to these numerous obstacles. An essential player tactic is to avoid contact with B Lymphocytes early in the game, which in turn delays the production of plasma cells that produce the billions of antibodies. Antibodies are the greatest threat of all, but game play can continue with increasing numbers of Killer T cells, it just gets harder. With each macrophage collision, the number of Killer T cells increases and the time period for replication decreases.

At the beginning of the game, there are dark, round cells known as Neutrophils in the circulation. Collision into macrophages results in the progressive replacement of Neutrophils with Killer T cells, which are larger and darker. Neither Neutrophils nor Killer T cells pose a direct threat to the player during migration. The threat comes during Cellular Attack, and during the immediate approach to Cellular Attack. Their relative proportions correspond to the amount of time available for each Infection. Being more aggressive, the increased presence of Killer T cells indicates that the time available for future Infections is decreasing.

If the virion does not collide into phagocytes or engage in Cellular Attack for a certain amount of time, complement begins to appear in the blood. These are small bundles of polypeptide chains, resembling knots, in the game. Colliding into complement causes it
to attach to the virion, slowing the virion down, increasing its turn radius and making it more vulnerable to macrophage pseudopods. Once complement appears in the game, it does not disappear until the game ends.

If the virion collides and accumulates three knots of complement, a countdown begins as the complement begins to eat away at the protein coat of the virion. If the virion does not succeed in beginning an Infection within the time limit, the protein coat disintegrates and the virion is inactive. The presence of complement forces players to survive by engaging in Cellular Attacks and reproducing rather than simply evading threats.

**Migration obstacles**

<table>
<thead>
<tr>
<th>Name of object</th>
<th>Appearance</th>
<th>Occurrence</th>
<th>Result of collision</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary wall</td>
<td>Narrow tunnel</td>
<td>Throughout</td>
<td>Nothing.</td>
</tr>
<tr>
<td>Venous wall</td>
<td>Convoluted tunnel</td>
<td>Throughout</td>
<td>Controlled bounce.</td>
</tr>
<tr>
<td>Arterial wall</td>
<td>Smooth tunnel</td>
<td>Throughout</td>
<td>Loss of control.</td>
</tr>
<tr>
<td>Red blood cell</td>
<td>Toroid without a hole</td>
<td>Throughout</td>
<td></td>
</tr>
<tr>
<td>Monocyte</td>
<td>Round, yin-yang nucleus</td>
<td>Throughout</td>
<td>Death. Move by chemotaxis towards infected sites, turning into macrophages.</td>
</tr>
<tr>
<td>More monocytes</td>
<td>Proliferate at site of infection</td>
<td>After 1 Infection</td>
<td></td>
</tr>
<tr>
<td>Macrophage</td>
<td>Amorphous, large, amoebic movement</td>
<td>After 3 Infections</td>
<td>Death. Killer T cells become sensitized.</td>
</tr>
<tr>
<td>B Lymphocyte</td>
<td>Round, large, shiny</td>
<td>Rare, increasing</td>
<td>Release of antibodies.</td>
</tr>
<tr>
<td>Antibody</td>
<td>Tiny, shiny T-shaped stars</td>
<td>Follows collision with B Lymphocyte</td>
<td>Formation of antigen-antibody complex and death.</td>
</tr>
<tr>
<td>Complement</td>
<td>Dark knots</td>
<td>Lack of collision or Infection</td>
<td>Loss of maneuverability.</td>
</tr>
<tr>
<td>3 Complement</td>
<td></td>
<td></td>
<td>30 second countdown to death.</td>
</tr>
<tr>
<td>Killer T cells</td>
<td>Round, large, dark</td>
<td>Follows collision with macrophage</td>
<td></td>
</tr>
</tbody>
</table>

**Inflammatory Response**

After two successful Infections of an organ, the tissue around the organ will become inflamed. This will have two effects on the game. Firstly, vasodilation causes the capillaries to enlarge, making capillary transit faster. This will make it more difficult for the player to initiate Cellular Attack. Secondly, increased permeability of vessel walls allows phagocytes, antibodies, *et cetera*, to enter the area more easily. Thus, players will face greater challenges trying to infect an already-infected organ.
**Fever and Host Death**

After each successful Infection, the temperature of the entire host body increases, representing a Fever. The player can see this information in the form of a thermometer on the side of the screen. Beyond a certain temperature, the playing screen will take on a pulsating red tint and the sound of the heartbeat will increase in rate. Consequently the speed of migration increases because the propulsion/transit time increases. The rate of progeny synthesis during Infection will drop when fever is present.

The temperature will increase with every successful Infection and will decrease if the player does not infect any cells over a certain period. If the temperature remains above 39.5 degrees centigrade for over a minute of play, then the host will die and the game will end. If the player allows the temperature to drop to a safe level, the systemic effects will cease and progeny synthesis will return to a normal rate, although local inflammation may remain in effect.

**Cellular Attack**

Once the player successfully navigates close to a target cell or clump of cells, a pair of crosshairs appears on the target cell. When the player presses ‘A’, the game shifts into Cellular Attack mode. The camera remains locked behind the virion, pointing directly towards the center of the cell. The player can use the left thumb stick to move left and right, up and down while facing the center of the cell, moving across the surface of the cell. The left and right triggers will rotate the camera on its axis. If the target cell is moving, the virion will move along with the target cell in the circulation. The virion will still be vulnerable to all the usual threats during migration even if the camera is not pointing in the direction of travel. Thus, it is in the player’s interest to enter the cell as soon as possible.

The virion needs to penetrate the cell wall before it actually begins replication inside the cell. If the virion is non-enveloped, i.e. during customization the player chose not to give the virion a lipid membrane, the virion enters the cell automatically and begins Infection. If the virion is enveloped, the player needs to skim the surface of the cell until it finds a receptor, which is brightly colored. Once it touches a receptor, the virion attaches securely to the cell surface and enters the cell. As a virion enters the cell, it loses its lipid envelope and protein coat.

The virus is now inside the cell and needs to replicate before the Killer T cells or the neutrophils destroy the infected cell. A countdown begins, based on the amount of time available before a Killer T cell or a neutrophil duly arrives. A shadow looms across the cell as the countdown approaches expiration. Macrophages also engulf the cell during Cellular Attack.

The player is now in control of the nucleic acid of the virus. In the case of Type VII viruses, the player controls one fragment of the virus DNA released into the cell. The player needs to move around the cell to gather the components necessary to synthesize progeny viruses. Most of the cell is cytoplasm and the player cannot leave the exterior
cell membrane. Somewhere near the center of the cell is the nucleus, which the player can enter and leave by finding one-way intracellular transport structures, resembling translucent pipes in the cell. In effect, the player is engaged in a free-roaming, spherical navigation game of chutes and ladders.

The player collects components simply by moving over them. The game will not pick up components that are not necessary. Several types of viruses will automatically begin replicating when the player collects enough enzymes or proteins so that beginning players will have less difficulty with Cellular Attack. These viruses do not yield good scores for replication. Others will require the player to press the ‘A’ button to activate the collected components, and Type VII viruses need to collect all the fragments of viral DNA before it can even begin to synthesize progeny. Type VI retroviruses require the player to press ‘A’ inside the nucleus in order to synthesize progeny.

Proteins are small, passive objects in the cell, whereas enzymes are larger and mobile. Proteins, DNA and enzymes have colors similar to the virus nucleic acid if they come from the virus, and different if they come from the cell. Most of the components contributed by the virus will be in the cytoplasm of the cell. Cell proteins are plentiful in the cytoplasm of the cell, whereas cell enzymes are easier to find in the nucleus. The process of viral assembly does not require the player to control any biochemical action of messenger RNA. mRNA operates automatically once the player has gathered the necessary enzymes to produce mRNA.

**Cellular Attack goals based on the Baltimore Classification of viruses**

<table>
<thead>
<tr>
<th>Type</th>
<th>Nucleic Acid</th>
<th>Collect</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>d/s DNA</td>
<td>Nothing</td>
<td>Automatic replication (Easiest type, lowest score)</td>
</tr>
<tr>
<td>II</td>
<td>s/s +DNA</td>
<td>Cell Proteins</td>
<td>Automatic replication after collecting 3 random proteins</td>
</tr>
<tr>
<td>III</td>
<td>d/s RNA</td>
<td>Virion Enzymes</td>
<td>Automatic replication after collecting virion enzyme</td>
</tr>
<tr>
<td>IVa</td>
<td>s/s +RNA</td>
<td>Cell Enzyme</td>
<td>Collect cell enzyme, ‘A’</td>
</tr>
<tr>
<td>IVb</td>
<td>s/s +RNA</td>
<td>Cell Enzymes</td>
<td>Collect 2 different cell enzymes, ‘A’</td>
</tr>
<tr>
<td>Va</td>
<td>s/s –RNA</td>
<td>Enzymes</td>
<td>Collect cell or virion enzyme, ‘A’. Do both to replicate.</td>
</tr>
<tr>
<td>Vb</td>
<td>s/s –RNA</td>
<td>Enzymes</td>
<td>Collect 1 cell and 1 virion enzyme, ‘A’.</td>
</tr>
<tr>
<td>VI</td>
<td>s/s +RNA</td>
<td>Virion Enzymes</td>
<td>Collect 3 different virion enzymes, ‘A’ inside the nucleus.</td>
</tr>
<tr>
<td>VII.1</td>
<td>d/s DNA</td>
<td>Viral DNA</td>
<td>Collect all fragments of Virus DNA, ‘A’. Go to VII.2</td>
</tr>
<tr>
<td>VII.2</td>
<td>d/s DNA</td>
<td>Enzymes, Proteins</td>
<td>Collect 2 different enzymes and 3 random proteins, ‘A’.</td>
</tr>
</tbody>
</table>
**Release of Progeny**

Progeny viruses re-enter the blood stream either by budding or by causing the infected cell to explode (lysis). Players may choose one of the two options while customizing their virus. Viruses that release progeny by budding automatically have their completed progeny enter the blood stream. Even if the time limit expires, all progeny synthesized thus far remain in the game. Budding yields a significantly lower score than lysis.

If players pick lysis as their preferred form of progeny release, they must press ‘B’ to make the infected cell explode after synthesizing progeny. If the time limit runs out before the player presses ‘B’, all synthesized progeny are lost as the immune system of the host performs phagocytosis on the infected cell. The player receives large score bonuses for each synthesized progeny released via lysis.

Upon the death of the cell, via lysis or phagocytosis, the player assumes control of one of the progeny viruses and begins migration again. The original infecting virion always dies at the end of an Infection. Released progeny during an Infection proportionally increases the number of virions in other parts of the host, whereas phagocytosis that interrupts lysis will proportionally reduce the number of virions in the host.

**Unlockable Missions**

The player can unlock different game missions after demonstrating mastery in the main game. These variations on the main game have slightly different goals for success. The player may select between missions in the same way that the player chooses attributes for the virus.

<table>
<thead>
<tr>
<th>Mission</th>
<th>Conditions to unlock</th>
<th>Game goals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host is immune</td>
<td>Successful play in main game for 5 minutes or more.</td>
<td>Game starts with large numbers of antibodies, memory cells, and a short time limit for Infection. Extra transmission bonus.</td>
</tr>
<tr>
<td>Inferno</td>
<td>Player manages to reduce temperature after fever.</td>
<td>Entire game with Fever conditions. Temperature does not decrease but the host will not die.</td>
</tr>
<tr>
<td>Medication</td>
<td>Successful infection while attacked by Complement.</td>
<td>Entire game with lowered maneuverability. Cellular Attack features reduced numbers of receptors.</td>
</tr>
<tr>
<td>Body Count</td>
<td>Player ‘achieves’ host death.</td>
<td>Kill as many hosts as possible within a fixed time limit. After host death, the player restarts in a new host.</td>
</tr>
</tbody>
</table>

**Multiplayer mode**

It is possible to play this game with more than one player at a time. If played on a console, the screen divides into 2, 3 or 4 parts with each player controlling a separate virus in each part of the screen. If played over a network, the screen is similar to individual play. The scores of other players appear alongside the player’s score. Progeny and target sites of other players appear in the same color as the score of the respective player, and all player-controlled virions have a slight glow. When one virus collides into
another, the virus that was moving slower in a lateral direction loses control for a second. Because uncontrolled virus progeny do not ‘steer’ in the same way as player-controlled virions, this only applies when two player-controlled virions collide into each other, though only a lateral collision would be possible.

The players may opt to play the same virus or different viruses in the same body. The players may also opt to play cooperatively (combined score) or competitively (collective scores). Unique power-ups become available in multiplayer mode, which allow players to aid or sabotage the progress of other players during the game. For instance, the player may be able to release antihistamines or interferon near opponents’ target cells, respectively helping and hurting the progress of his opponents. If the players choose to play the same virus in cooperative mode, when one virus mutates, all of them mutate at the same time.
Content Notes and implications for gameplay

Methods of Viral Evasion / Subversion of the Immune Response

• Infection of Immunologically Privileged Sites

In this strategy, the virus infects cells which are not easily accessed by the immune system.

- Central Nervous System due to the blood-brain barrier through which lymphocytes cannot get. Additionally, neurons are cells which cannot be directly recognized by killer T cells.

- Kidneys for which the reason is not fully understood, though T cells have limited access to infected epithelial cells in the kidney, due to the presence of an intact basement membrane. Eg. Cytomegalovirus.

- Epithelial surfaces of other secretory or excretory glands, including the salivary gland (favoured by Cytomegalovirus and Epstein Barr virus).

- Epidermis (comprised of keratinocytes) in which the infected cells are separated from the host’s immune response by a basement membrane. Eg. Papillomavirus.

While present in the above types of cells, the viruses are said to persist, and during this time, viruses with RNA genomes are able to undergo mutation. Mutation is a way of evading both T and B cell immunity.

• Viral Escape from Antibody Recognition

Alteration of viral proteins at sites critical for antibody recognition - epitopes. Eg. Antigenic shift and drift seen in Influenza. Antigenic changes occur as a result of alterations in the 2 surface glycoproteins. Seen primarily in RNA viruses.

Molecular Mimicry
Viral antigens mimic epitopes (determinant sites) present on host proteins. Eg. Measles and Cytomegalovirus. This triggers the production of antibodies against the ‘self’ proteins, resulting in autoimmune diseases.

• Viral Escape from T Cell Recognition

Suppression of Cell Surface Molecules Required for T Cell Recognition.

Down regulation of the expression of host molecules which are necessary for effective T cell recognition of infected host cells.
• Inhibition of cellular apoptosis (apoptosis being a normal part of cellular activity).

• Restricted gene expression – virus remains latent in the cell with minimal or no expression of viral proteins. Eg. Herpes Simplex Virus in neurons, Epstein Barr Virus in B cells, Human Immunodeficiency Virus in resting T cells.

• Defense molecules which interfere with the function of antiviral cytokines.

**Virus Entry Mechanisms**

The role of the virion is to deliver the nucleic acid to the host target cells. The surface of each virus has receptors which bind to the target cell. Once bound to the cell surface, the virus gains entry in a number of ways.

• Non-enveloped viruses are internalized by the cell itself – a process of translocation across the membrane, or else the membrane invaginates to form a vacuole (endocytosis). The vacuole is often acidified by proton pumps in the membrane or by fusion with digestive endosomes, but some viruses have proteins which, in the lower pH (acidity) are able to mediate the exit of the viral genome into the cell. Eg. Reoviruses, and Adenoviridae.

• Hydrophobic regions of outer proteins interact with cellular membrane and allow the genome itself to penetrate the cell. Eg. Picornaviridae.

• Enveloped viruses are internalized in vacuoles and fuse with the vacuole wall due to effect of acidic environment on outer proteins. Eg. Orthomyxoviridae (Influenza) and Togaviridae. Some members of Rhabdoviridae and Retroviridae.

• Fusion of virion to cell surface and release of nucleocapsid directly into cytoplasm. Eg. Paramyxoviridae and Herpesviridae. Some members of Retroviridae (including HIV).

**Replication and Viral Genome Types**

Following cell entry (with exception of latency) the first event of replication is the eclipse phase during which the virus fragments in order to begin replication, but has not yet assembled the components of progeny virus. This period can range from a few hours to a few days, but is shorter with bacteriophages. Next, progeny virus genomes assemble into new viruses, which in turn make more viruses, so the cell becomes dedicated to virus production. This is the logarithmic phase. Cell death then follows. The logarithmic phase is referred to as secondary transcription.

The viral genome influences the replication strategy.

• **Double Stranded DNA** - Large in size.

  Poxviridae  Herpesviridae  Adenoviridae
Smallpox   Herpes Simplex   Common Cold
          Epstein Barr
          Cytomegalovirus

- **Single Stranded DNA**

  **Paroviridae**
  - B19 Infection

- **Double Stranded RNA** - Usually smaller than DNA genomes.

  **Reoviridae**
  Rotavirus

- **Single Stranded RNA** - Subdivided into: mRNA (positive sense) and those which are complementary to the mRNA produced from them (negative sense).

  **Positive sense:**
  - Picornaviridae
  - Caliciviridae
  - Coronaviridae
  - Flaviviridae
  - Polio
  - Norwalk
  - Common Cold
  - Yellow Fever

  **Togaviridae**
  Rubella

  **Negative sense:**
  - Orthomyxoviridae
  - Paramyxoviridae
  - Rhabdoviridae
  - Filoviridae
  - Influenza
  - Measles, Mumps
  - Rabies
  - Ebola

- **Viruses with RNA genomes which use a DNA intermediate stage to produce the RNA genome.**

  **Retroviridae**
  Human Immunodeficiency Virus

- **Viruses with DNA genomes which use an RNA intermediate stage to produce the DNA genome.**

  **Hepadnaviridae**
  Hepatitis B

**Specific Examples:**
**Herpesviridae**

Virions are spherical or pleomorphic. Diameter 150 – 200 nm. Consist of (1) envelope with surface projections (2) tegument made of amorphous material (3) an icosahedral nucleocapsid (4) a core consisting of a fibrillar spool on which the DNA is wrapped.

Genome consists of a single molecule of linear, double-stranded DNA.

Replication starts with circularization of viral DNA. Transcription and translation are regulated and sequentially ordered in a cascade with 3 major stages. Immediate early genes are transcribed by nuclear enzymes. And mRNAs are transported to the cytoplasm and translated. Proteins are then transported to the nucleus and are involved in the synthesis of additional mRNAs. Early proteins are involved in the replication of viral DNA by a rolling circle mechanism. Late mRNAs are translated mostly into structural proteins. Replication takes place in the nucleus and capsids acquire their envelopes via budding through the inner lamella of the nuclear envelope. Virions are released via transport across the cytoplasm in membranous vesicles which then fuse with the plasma membrane.

Transmission is by contact – infected cells in saliva, urogenital excretions and free virus in aerosols. Some viruses induce neoplasia. Many persist for the lifetime of the host and exhibit latency.

Virus is sensitive to acid, heat.

**Paramyxoviridae**

Virions are 150 – 300 nm in diameter, usually spherical and with a lipid containing envelope, with large peplomers 8 – 12 nm in length, within which is coiled a helical nucleocapsid. Nucleocapsid is 13 – 18 nm in diameter, up to 1,000 nm in length, with a 5.5 – 7 nm pitch.

Genome consists of a single molecule of linear, negative sense, single stranded RNA. Surface glycoproteins of some viruses have neuraminidase activity, hemagglutinating activity, and fusion activity.

Replication. RNA replication involves mRNA transcription from the genomic RNA via the virion transcriptase. Using the protein products of this transcription there is production of full-length positive stranded template, which is then used for the synthesis of genomic RNA. The genome is transcribed processively by virion associated enzymes into 6 – 10 separate, sub-genomic, viral complementary mRNAs. The mRNAs are capped and have a 3’-poly (A) tract. Replication takes place in cytoplasm, and assembly occurs via budding on plasma membranes. They have a narrow host range.

Transmission is horizontal by aerosols and droplets.
Virions are sensitive to heat, lipid solvents, oxidizing agents.

References


Content Notes on Immunology

Numbers are per cubic millimeter

Blood
45% Formed Elements

• Erythrocytes (red blood cells) 4.8 – 5.4 million

• Leucocytes (white blood cells) 5,000 – 10,000
  - Neutrophils (60 – 70%)
  - Eosinophils (2 – 4%)
  - Basophils (0.5 – 1%)
  - Lymphocytes (20 – 25%)
  - Monocytes (3 – 8%)

• Thrombocytes 250,000 – 400,000

55% Plasma

• Proteins (7%)
  - Albumins (55%)
  - Globulins (38%)
  - Fibrinogen (7%)

• Water (91.5%)

• Other solutes (1.5%)

Phagocytosis
Neutrophils have the greatest phagocytic role. Granulocytes (neutrophils) and monocytes migrate to an infected area. During migration the monocytes develop into larger phagocytic cells called macrophages. There are fixed macrophages which reside in certain tissues and organs. The wandering macrophages leave the blood and migrate to infected areas.

4 Stages of Phagocytosis

• Chemotaxis - the chemical attraction of phagocytes to microorganisms. Chemotactic chemicals are microbial products, components of white blood cells, damaged tissue, and chemicals derived from complement.

• Adherence - the attachment of a phagocyte cell membrane to the surface of a microorganism. Easy attachment occurs when the microorganism is first coated with certain plasma proteins (complement) which promotes adherence. Difficult attachment is facilitated by the phagocyte trapping the particle against a rough surface such as connective tissue fibres, a blood vessel or clot.
• Ingestion - the phagocyte’s cell membrane extends projections called pseudopods which engulf the organism. The pseudopods surround, meet and fuse around the organism creating a sac called the phagocytic vesicle.

• Digestion - phagocytic vesicle separates from the cell membrane and enters the cytoplasm. The cytoplasm contains lysosomes. The phagocytic vesicle and lysosome membranes fuse to form a phagolysosome or digestive vesicle.

Though most microorganisms are killed at the last stage, some, such as staphylococci (bacteria), are ingested but not killed. Others multiply within the phagolysosome, such as tubercle bacillus, eventually destroying the phagocyte. Some organisms remain dormant.

**Inflammatory Response**
This is a defense mechanism in response to bacterial or viral invasion.

• Vasodilation and increased permeability of blood vessels. Vasodilation allows more blood to reach the affected area. Increased permeability allows phagocytes, antibodies, *et cetera*, to enter the area. Increased blood flow also helps to remove toxic substances produced by microorganisms.

• Substances involved in the inflammatory response are:

  Histamine. Phagocytes, attracted to the site, stimulate the release of histamine from cells such as basophils and blood platelets. It is histamine which produces the vasodilation and increased permeability.

  Kinins serve as chemotactic agents for phagocytes. Kinins are polypeptides present in the blood which produce vasodilation and permeability.

  Prostaglandins are released by damaged cells and intensify the effects of histamine and kinins. They also stimulate the migration of phagocytes through the capillary walls.

  Leukotrienes are produced by basophils and mast cells. They function in adherence, chemotaxis and they increase permeability.

  Complement. A group of plasma proteins which stimulate the release of histamine by mast cells, basophils and platelets. They attract neutrophils by chemotaxis. They promote phagocytosis and can destroy bacteria.
• Fibrin Formation
Increased permeability of capillaries causes fibrinogen (a soluble protein) to leak into the tissues where it is then converted into insoluble fibrin which traps invading organisms.

• Phagocyte Migration.
The inflamed tissue produces a substance called leucocytosis promoting factor. This ensures a steady production and release of neutrophils in the bone marrow. As flow of blood decreases, the neutrophils stick to the inner surface of the endothelium of blood vessels = margination. They squeeze through the wall of the blood vessel to reach the site. This migration to the site is called diapedesis. This movement depends on chemotaxis as a result of the presence of other neutrophils, complement, and kinins.

• Neutrophils also contain antibiotic type chemicals called defensins which are active against bacteria, fungi and viruses.

• Next, monocytes follow the neutrophils into the affected area. On entering the tissue, monocytes become wandering macrophages. Neutrophils perform the early phagocytosis, and then the macrophages take over once the neutrophils die.

• Macrophages are much more phagocytic than neutrophils, and larger. They can engulf tissue, dead neutrophils and invading microbes.

Pyogenesis

The formation of pus, containing living and non-living white blood cells and debris, is normal. It escapes from the body via an internal cavity for dispersal. If pus forms in a confined space and cannot escape, then an abscess can develop. Sometimes pus is destroyed and absorbed by the body.

Fever/Pyrexia
Fever is a response to infection by viruses or bacteria. When monocytes and macrophages ingest bacteria, the cell wall of the bacterium is released, stimulating the phagocytes to secrete interleukin-1. The interleukin-1, in turn, reaches the anterior hypothalamus where it induces neurons in the preoptic area to produce prostaglandins.

The prostaglandins cause the hypothalamic thermostat to be reset at a higher temperature. Following this, reflex mechanisms act to bring the core temperature up to a new level. Aspirin, acetaminophen and ibuprofen inhibit the synthesis of prostaglandins, thus reducing temperature.

Benefits of fever:

• Fever is beneficial and interleukin-1 helps promote the production of T-cells.
• Raised temperature increases the effect of interferon.

• Inhibits the growth of some bacteria and viruses.

• Antibody production and T-cell proliferation increase.

• Chemical reactions speed up.

**Antimicrobial Agents – Interferon, Complement and Properdin**

• Interferon is a protein produced by cells when they are infected by viruses. Three types of interferon – alpha, beta and gamma. Interferon is produced by lymphocytes, some other leucocytes and fibroblasts.

When a virus-infected cell releases interferon, the interferon diffuses to other nearby cells which are not infected, and binds to the surface receptors. These uninfected cells begin to synthesize antiviral proteins which inhibit viral replication. Interferon is important in the body’s defense against viruses, because viruses can only cause disease if they can replicate within cells.

• Complement = approx 20 proteins normally in the blood serum. These complement the action of antibodies. The antibody forms an antigen-antibody complex and activates complement for attack. The antigen-antibody complex causes the complement to attach to the invading microbe.

Effects of attached complement:

- **Cytolysis.** Certain complement proteins cause reactions which create holes in the plasma membrane of the microbe. The contents then leak out.

- Promotion of the release of histamine from mast cells, basophils and platelets.

- Assist in the process of chemotaxis.

- **Opsonization or Immune Adherence.** Complement proteins bind to the surface of a microbe and communicate with receptors on phagocytes.

• Properdin. A complex of 3 proteins found in serum, involved in cytolysis, the enhancement of phagocytosis, and in the inflammatory response.

**Antigen-Antibody Response**

An antigen is a substance which stimulates the body to produce antibodies or T cells.
A microbe, such as a bacterium or virus, is antigenic, as are bacterial structures (flagella or cell walls, for instance) and bacterial toxins.

Some antigens are nonmicrobial, such as egg white, pollen, transplanted organs, incompatible blood cells (given as transfusion). Chemically, antigens are proteins, nucleoproteins (nucleic acid + protein), lipoproteins (lipid + protein), glycoproteins (carbohydrate + protein), or polysaccharides.

Antigens are recognized by the body as non-self, as foreign. On the surface of the antigen are antigenic determinant sites upon which specific chemical groups of the antigen combine with the antibody. The number of antigenic determinant sites on the antigen surface is referred to as the valence. Many antigens are multivalent, but an antigen must have at least two determinant sites in order to induce antibody formation.

An antibody is a protein which is capable of combining with a specific antigen, the presence of which stimulates the body to produce the antibody in the first place. Most human antibodies are bivalent. The antibody corresponds with the antigen in a lock and key analogy. Antibodies are also known as immunoglobulins or Ig, because they belong to a group of proteins called globulins.

Five classes of immunoglobulins:

- IgG antibodies are the most abundant, found in blood, lymph and the intestines. Their action against bacteria and viruses includes enhancement of phagocytosis, neutralization of toxins, and triggering of the complement system.

- IgA antibodies are found in tears, saliva, mucus, milk, blood, lymph, and secretions from the gastrointestinal tract. They provide local protection on mucous membranes.

- IgM antibodies are always the first antibodies to appear in response to an antigen. Found in blood and lymph and on the surface of B lymphocytes. They cause agglutination and lysis of microbes.

- IgD antibodies are located in blood, lymph and on the surface of B lymphocytes and they stimulate antibody-producing cells to produce antibodies.

- IgE antibodies are located on mast and basophil cells – involved in allergic reactions.

The antibody molecule assumes the shape of the letter 'Y' or the letter 'T'. Antibodies are comprised of two pairs of identical polypeptide chains. The heavy (H) chains consist of 400 or more amino acids and the light (L) chains each have approximately 200 amino acids. Please see Figure 1. The tops of the H and L chains, called variable portions, contain the antigen binding sites. The variable portion is different for each antibody and this allows the antibody to recognize and attach to a specific kind of
antigen. Antibodies with two variable portions for attachment to antigen are bivalent. The remainder of the polypeptide chain is the constant portion. It is the constant portion which is the same in antibodies of the same class and determines the type of antigen-antibody reaction which occurs.

Prior to combination with the antigen, antibodies resemble the letter 'T', but afterwards they resemble the letter 'Y' and are smaller. An antigen-antibody complex is formed.

**T Lymphocytes and Cellular Immunity**

Macrophages (phagocytes derived from monocytes) process and present antigens to T cells. The antigen is phagocytized by the macrophage and, once partially digested, is presented on the surface of the macrophage together with human leucocyte associated (HLA) antigen. Receptors on the T cell recognize this. The T cell is now sensitized. While processing antigen, macrophages secrete lymphokines - interleukin-1 and interferons, which stimulate T cell growth.

There are thousands of different types of T cells, each of which responds to a specific antigen or group of antigens. When an antigen invades the body, the one particular T cell which deals with that particular antigen becomes activated. Cell division occurs and the T cell clones itself, forming subgroups of the exact same T cell. The subgroups are as follows, according to function:

- Cytotoxic (killer) T cells – migrate from the lymphoid tissue to the site of invasion, where they attach to the invading microbe and secrete a lymphokine called lymphotoxin, which destroys the antigen. Other lymphokines secreted by killer T cells are:
  - Perforin, which perforates a cell membrane causing lysis of the cell.
  - Transfer factor reacts with nonsensitized lymphocytes at the site, causing them to take on the characteristics of the cytotoxic T cells.
  - Macrophage chemotactic factor attracts macrophages to the site.
  - Macrophage activating factor increases their phagocytic action.
  - Macrophage migration inhibition factor.
  - Mitogenic factor causes nonsensitized lymphocytes to divide faster.

Summary: Cytotoxic Killer T cells secrete lymphotoxin and other lymphokines, attract macrophages, intensify phagocytosis. Additionally they produce interferons which inhibit viral replication.

- Helper T cells - work with B lymphocytes to increase antibody production.
Helper cells interact with antigen, thus inducing antibody production by the descendants of B lymphocytes. Helper T cells secrete interleukin-2, a lymphokine which stimulates the proliferation of cytotoxic T cells. Helper T cells secrete proteins which increase the intensity of the inflammatory response, and promote killing by macrophages.

- Suppressor T cells - a few weeks after the initial invasion, suppressor T cells dampen the immune response by shutting down certain activities. They inhibit the production of antibodies by plasma cells, et cetera.

- Delayed hypersensitivity T cells - the source of several lymphokines involved in the hypersensitivity (allergy) response, and the rejection of transplanted organs.

- Amplifier T cells – stimulate helper and suppressor T cells, and descendants of B cells to higher levels of activity.

- Memory T cells – if the pathogen invades the body at a later date, the memory T cells remember and recognize the antigen, and initiate a very fast and overpowering immune reaction.

- Natural Killer cells – these are lymphocytes but differ from the killer T cells. They lyse certain target cells by releasing the lymphotoxin perforin. They attack cancer cells.

**B Lymphocytes and Humoral Immunity**

When a foreign antigen invades, B cells in the lymph nodes, the spleen, and the gastrointestinal tract become activated, and so they differentiate into antibody producing plasma cells.

The antigen binds to antibodies on B cells. The antigen is processed and presented on the surface of B cells along with HLA antigen. The helper T cells then recognize this antigen. The helper T cells produce substances which promote B cell division. Some B cells transform into plasma cells which produce the antibodies. Interleukin-1 helps this process.

Rate of antibody production = 2000 molecules per second for each plasma cell. This continues for 4 – 5 days until the cell dies. Remaining B cells turn into memory B cells, which serve a similar role as memory T cells.

There are thousands of different B cells, each type specific to a certain antigen. A given antigen stimulates the action of only those B cells predetermined to produce the correct antibody.

Antibodies produced by B cells form antigen-antibody complexes with foreign antigens.
T cells and B cells are located in the lymphoid tissue in the spleen, lymph nodes, gastrointestinal tract and bone marrow.

**Primary Immune Response and Anamnestic Response**
The second or subsequent exposure to an antigen produces a more intense immune response than the initial exposure. During the primary response to the initial exposure, the body is sensitized. Immunocompetent lymphocytes proliferate. When the antigen is introduced again, there is an immediate reaction and the production of antibodies. Some of the lymphocytes from the first exposure remain as memory cells, thus exacerbating the secondary or anamnestic response significantly.

**Translation to the game:**
- *Interferon* will interfere with the player’s ability to enter and replicate in *some* cells. Hence, speed in the early stages of the game is essential. As time progresses, more and more neighbouring cells will have synthesized antiviral proteins. Because interferon binds to the surface receptors of *nearby* uninfected cells, the player is more likely to successfully replicate inside cells which are some distance from other already infected cells. Therefore, if the virus in question only attacks a very localized and particular group of cells, then this becomes difficult. Contrariwise, if the virus has an ability to attack a more generalized type of cell, it becomes easier for the player to find interferon-free cells.

- *Neutrophils* and *monocytes* migrate to the site of infection, and therefore can be active and move around in menacing clusters in the game.

- *Monocytes* turn into *wandering macrophages* which are larger and scarier and capable of engulfing the virus / player.

- The neutrophils will attack first, followed by the macrophages which are far more sinister than neutrophils.

- If the virus / player gets covered in *complement* then the macrophages will be able to attach to the player much more easily. This is clearly bad news for the player. If the player has avoided getting covered in complement, there is a chance of wriggling free from a macrophage through speed and dexterity. Complement also makes the microbe / virus develop holes in its protein coat, which will ultimately lead to the loss of fragments of the precious nucleic acid.

- The *inflammatory response* creates permeability of the blood vessels. It might be appropriate to have some gaps or holes in the sides of the blood vessels through which the player is travelling. Throughout the course of the game, these could either enlarge and / or increase in number, to correspond with increasing inflammation. It will be a great disadvantage to a player if he or she accidentally pops out through a hole in the wall. Good steering will help to limit the risk of this, but the more holes the harder it becomes. Likewise, the area of inflammation
will be an area of increased blood flow, thus pulling the player (by now frantic) at speed through a dangerous area with many holes.

- The player / virus may seek temporary refuge by attempting to enter a suitable cell, in order to replicate, but in a short space of time a cytotoxic killer T cell will come and attack by secreting lymphotoxin and perforin.

- Antibodies will start attaching to each antigen /virus in the game, turning them into complexes, and making them useless. The magnificent speed of antibody production will mean that the player has to keep on replicating in order not to get overpowered. The antigen-antibody complex is destroyed by a phagocyte.

- The player can delay the sensitization and proliferation of killer T cells by avoiding getting engulfed by a macrophage. Likewise he can delay the production of plasma cells which produce billions of antibodies by avoiding any contact with a B Lymphocyte. Macrophages and B Lymphocytes “process and present” the antigen, stimulating the proliferation of cytotoxic killer T cells and plasma cells respectively. The player can prolong the game and replicate more effectively by using these tactics. Despite this, the player will ultimately succumb to the immune response, unless he is particularly virulent or able to mimic other cells thus disabling recognition.
**Pedagogy**

Understanding the role of the immune system in the body’s fight against viral infection is a standard concept in the Biology curricula of many high schools and colleges.

Traditionally, the concepts have been taught in textbooks, lectures, diagrams, and films. For obvious reasons, hands-on experiments with viruses are impossible in a classroom setting. Thus, the study of viruses and the immune system response has become an abstract concept for many students, despite its enormous impact on contemporary life and its starring role in epidemics.

Science and medical educators are realizing that the traditional didactic approach to teaching immunology has very clear limitations. Educators are turning instead to in-depth case studies and simulated problem-solving (Barrows et al, 1999). While *Replicate!* is not a conventional case-study, the game play does include many of the same elements. Case study problems are described in text or diagrams, but virtual environments allow designers to embed problems within the context of the game. Exploring a 3-dimensional simulation of the human body offers an engaging way to learn, as does the novelty of being the bad guy - the virus.

*Replicate!* does not replace a textbook or teacher, but supplements traditional instruction to clarify the process of viral infection and immune response, in a fun, breathtaking action game. The game literally allows students to visualize how viruses spread. This ability to visualize microscopic phenomena is a characteristic that scientists often notice within themselves, but is often found to be lacking in students who have trouble with science. In this respect, the game encourages “disciplinary” thinking, allowing students to see how scientists see.

Students come to a classroom with preconceptions about how the world works. If their initial understanding is not engaged, they may fail to grasp the new concepts and information taught, or they may learn facts for the purposes of a test but revert to their preconceptions outside the classroom (National Research Council, How People Learn: Bridging Research and Practice, 10).

*Replicate!* attempts to solidify their knowledge by placing students inside the process of viral infection. Rather than rote memorization of facts, students play in an environment that rewards their understanding of the content, and emphasizes success at the game as a motivation for learning. Furthermore, by engaging with the virtual environment, students will confront their own incorrect preconceptions about the viral infection process and immune response, and be compelled to discover how to change these misconceptions in order to win the game.

Child psychologist Jean Piaget described a model of children as builders of their own intellect, capable of learning without being specifically taught via formal instruction. By taking on the role of a virus with the goal of infecting a target, students learn first-hand how the process of viral infection works. While a successful infection will often require a
linear path, students need to discern that path through the scaffolding of the game. In this way, they become familiar with the body’s circulatory and immune systems.

In addition, the game offers players an opportunity to “construct” their own, unique, virus, based on characteristics of others found in the game. Changing variables will affect the behavior and abilities of the virus, as well as the body’s response to it. Through the construction of an original virus, a player will have the opportunity to experience their own empirical observation, and test out their virus in a digital environment - a never before possible learning tool. Players can save their uniquely custom constructed viruses alongside the main selection of pre-existing viruses, so that other players can choose to play someone else’s virus, or challenge each other, and so on. The main selection includes Ebola, Influenza, Hepatitis B, Epstein Barr, Measles, Rubella, Polio, Common Cold, Smallpox, Rotavirus.
IMAGES OF VIRUSES
Following are images of viral attack / explanatory diagrams.

Figure 1.18: Hepatitis B virus replication.
Figure 1.15: Herpes simplex virus replication (dsDNA virus). In latent infection, late mRNAs and proteins are not produced, and circularized dsDNA is maintained as an episome with very limited transcription.
General Features

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History
In 1958 Dennis Burkitt described a lymphoma that represented the most common tumor affecting children in certain parts of East Africa. The geographical distribution of this malignancy suggested that the development of Burkitt’s lymphoma (BL) might be due to an infectious agent. In 1964 the successful establishment of cell lines from explants of BL enabled Epstein and Barr to identify herpesvirus-type particles by electron microscopy within a subpopulation of tumor cells in vitro. W. and G. Henle subsequently demonstrated that BL-derived cell lines expressed antigens that were recognized not only by sera from patients with BL but also by sera from patients with infectious mononucleosis (IM). Similar seroepidemiological studies also suggested a link between the so-called Epstein–Barr virus (EBV) and undifferentiated nasopharyngeal carcinoma (NPC) leading to the subsequent direct demonstration of EBV DNA in the tumor cells of NPC. The ability of this virus to efficiently immortalize B lymphocytes in vitro and to induce lymphomas in nonhuman primates established EBV as a putative oncogenic agent in humans. More recent studies have implicated EBV in a variety of other lymphoid and epithelial malignancies (Table 1).

Taxonomy and Classification
EBV is a member of the genus Lymphocryptovirus which belongs to the lymphotropic Gammaherpesvirinae subfamily of the family Herpesviridae. The lymphocryptoviruses exhibit a similar genomic structure and can infect the B lymphocytes of Old World primate species, resulting in latent infection often associated with lymphoproliferation. The 172 kbp EBV genome resembles other lymphocryptovirus genomes in that it contains (1) tandemly reiterated 0.5 kbp terminal direct repeats, and (2) tandemly reiterated 3 kbp internal direct repeats which divide the genome into unique short and long regions.

Geographic and Seasonal Distribution
EBV is ubiquitous being found as a widespread and largely asymptomatic infection in all human communities. Primary infection often occurs early in life particularly in tropical
areas and in persons of lower socioeconomic class. Thus, in tropical Africa and New Guinea primary EBV infection is common in the first year of life whereas in Western communities infection is continually acquired throughout childhood and early adulthood. Although the EBV-associated malignancies BL and NPC exhibit an unusual geographic distribution this appears not to be due to differences in EBV infection but to additional cofactors.

**Host Range and Virus Propagation**
Humans are the natural host for EBV infection. Certain nonhuman primates, particularly the cotton-top tamarin, have been used as experimental hosts for EBV but in these animals virus infection is associated with the induction of lymphomas. Lymphoblastoid cell lines (LCLs) generated by EBV immortalization of cotton-top tamarin B lymphocytes in vitro produce large amounts of virus compared with their human counterparts and as such have been used as a source of EBV, e.g. the prototype strain of EBV is B95.8 which is produced from a tamarin LCL originally immortalized with EBV from a patient with IM. However, the lack of a fully permissive system for propagating EBV in vitro has hampered our understanding of virus replication and prevented the generation of EBV mutants. New culture systems for efficient replication of EBV include anti-immunoglobulin-treated BL cell lines and epithelial cell lines transfected with the EBV receptor.

**Genetics**
EBV isolates from different regions of the world or from patients with different virus-associated diseases are remarkably similar when their genomes are compared by restriction fragment length polymorphism (RFLP) analysis. However, variations in repeat regions of the EBV genome are observed among different EBV isolates. Analysis of the EBV genome in a number of BL cell lines has revealed gross deletions in the viral genome some of which account for biological differences, i.e. P3HR-1 virus which is nontransforming contains a deletion of the EBNA 2-encoding gene.

Strain variation over the EBNA 2-encoding (BamHI WYH) region of the viral genome has permitted all EBV isolates to be classified as either ‘type 1’ (EBV-1, B95.8-like) or ‘type 2’ (EBV-2 Jijoye-like). This genomic variation results in the production of two antigenically distinct forms of the EBNA 2 protein which share only 50% amino acid homology. Similar strain-specific variation occurs in the EBNA 3-encoding region of the viral genome. These differences have functional consequences as EBV-2 isolates are less efficient in in vitro B lymphocyte transformation assays compared with EBV-1 isolates. Recent work demonstrates that sequence variation over the LMP1 gene, particularly a 30 bp deletion in the C-terminus of the protein, is a common feature of EBV isolates in certain regions (i.e. China) and may predispose to the development of virus-associated tumors.

**Evolution**
EBV, like other herpesviruses, has probably evolved with humans. The relatedness of the lymphocryptoviruses at both the genomic and protein levels does not correlate with the evolutionary relatedness of their Old World primate hosts. This implies that the selective
pressures governing the evolution of these viruses are different from those responsible for the evolution of primate species. It has been suggested that the specific tissue tropism of lymphocryptoviruses may have constrained their evolutionary divergence.

The evolutionary relationship between the type 1 and type 2 strains of EBV remains obscure. They may have evolved from a common progenitor virus or through recombination of either EBV strain with one of the lymphocryptoviruses infecting Old World primates. The pronounced (but not exclusive) segregation of EBV-2 isolates within equatorial regions suggests that environmental factors may have influenced EBV evolution and may still be responsible for the effective competition between EBV-2 isolates and the ubiquitous EBV-1 family.

Serologic Relationships and Variability
EBV isolates can be classified as type 1 or type 2 on the basis of allelic polymorphism of virus-encoded nuclear antigens EBNA1, 2, 3A, 3B and 3C. Both seroprevalence studies and typing of EBV in BL cell lines and LCLs have demonstrated that the majority of wild-type isolates in Western communities are of the EBV-1 strain whereas EBV-2 strains appear to be largely restricted to areas of equatorial Africa and New Guinea. However, in immunosuppressed HIV-positive individuals infection with both EBV-1 and EBV-2 can be found and EBV-2 is frequently detected in the lymphomas arising in these patients.

Further microheterogeneity exists among EBV isolates, particularly over the repeat regions of the viral genome. These give rise to variations in the size of the individual EBNA proteins which are apparent after their electrophoretic separation on polyacrylamide gels. Thus, the EBNA 1, 2, 3a, 3b and 3c proteins encoded by any one virus isolate display a unique size combination in gels which has been termed the ‘EBNA type’ of that particular isolate. This characteristic of EBV isolates can be used to trace the origin and transmission of EBV within families.

Epidemiology of EBV-associated Diseases

Infectious mononucleosis
Infection with EBV is widespread and, once infected, individuals become life-long virus carriers. Primary infection with EBV in childhood is usually asymptomatic but when delayed until adolescence or early adulthood can manifest clinically as IM, a self-limiting lymphoproliferative disease. The incidence of IM is low in Third World countries where primary infection predominantly occurs in childhood. In certain poorly defined situations IM-like symptoms can persist, resulting in chronic active EBV infection associated with elevated antibody titers to virus lytic antigens but low titers to the EBNAs.

Burkitt’s lymphoma (BL)
The endemic form of BL which is found in areas of equatorial Africa and New Guinea represents the most common childhood cancer (peak age 7–9 years) in these regions with an incidence of up to 10 cases per 100,000 people per year. This high incidence of BL is associated with holoendemic malaria accounting for the climatic variation in tumor incidence.
incidence first recognized by Dennis Burkitt. More than 95% of these endemic BL tumors are EBV-positive compared with 20% of the low-incidence, sporadic form of BL which occurs worldwide (Table 1). In areas of intermediate BL incidence, such as Algeria and Malaysia, the increased number of cases correlates with an increased proportion of EBV-positive tumors. A consistent feature of all BL tumors, irrespective of geographical location, is chromosomal translocations involving the long arm of chromosome 8 (8q24) in the region of c-myc proto-oncogene and either chromosome 14 in the region of the immunoglobulin heavy-chain gene or, less frequently, chromosomes 2 or 22 in the region of the immunoglobulin light-chain genes. Seroepidemiological studies have demonstrated elevated antibody titers to EBV capsid antigen (VCA) and early antigens (EA) in BL patients compared to children without the tumor. These elevated antibody titers have been found to precede the development of BL and can therefore be used to screen ‘at risk’ individuals.

**Nasopharyngeal carcinoma (NPC)**
The association of EBV with undifferentiated NPC was first shown by serological evidence and later confirmed by the demonstration of EBV DNA in NPC biopsy material. NPC is particularly common in areas of China and Southeast Asia reaching a peak incidence of around 20–30 per 100,000. Incidence rates are high in individuals of Chinese descent, irrespective of where they live, and particularly in Cantonese males. In addition to this genetic predisposition, environmental cofactors such as dietary components (e.g. salted fish) are thought to be important in the etiology of NPC. Extensive serological screening the EBV-specific antibody titers in high incidence areas, in particular IgA antibodies to EBV capsid antigen (VCA) and early antigens (EA), have proved useful in diagnosis and in monitoring the effectiveness of therapy. The association between EBV and undifferentiated NPC has been confirmed for many different racial groups, whether these exhibit a high, intermediate or low incidence of the tumor. Undifferentiated NPC is associated with a prominent lymphoid stroma and tumors arising at other anatomical sites (e.g. lung, stomach, salivary gland) with this morphology (lymphoepitheliomas) tend to be EBV-positive (Table 1). EBV infection is also associated with the more differentiated squamous cell NPCs particularly those occurring in the Far East. EBV infection has been detected in a proportion of common gastric adenocarcinomas.

**Lymphomas in patients with immunodeficiency**
Patients with primary immunodeficiency diseases such as X-linked lymphoproliferative syndrome (XLP) and Wiscott–Aldrich syndrome are at increased risk of developing EBV-associated B cell lymphomas. Because these tumors are extremely rare little is known of their association with EBV infection. Mortality from XLP is high with around 50% of patients developing fatal IM after primary infection with EBV and an additional 30% of patients developing malignant lymphoma.

Allograft recipients receiving immunosuppressive therapy and patients receiving immunosuppressive therapy patients with AIDS are also at increased risk for development of EBV-associated lymphoproliferative disease and lymphomas. The incidence of B cell lymphomas in allograft recipients varies with the type of organ...
transplanted and with the type of immunosuppressive regimen used. Allogeneic bone
marrow transplantation into EBV seronegative children is a particular risk factor for the
development of virus-associated B cell lymphomas.

The incidence of non-Hodgkin lymphoma in AIDS patients is increased approximately
60-fold compared to the normal population. Around 60% of these tumors are large-cell
lymphomas like those found in allograft recipients, 20% are primary brain lymphomas
and 20% are of the BL type. Recent studies have demonstrated that 50% of AIDS
lymphomas are EBV-positive and that this association varies with the histological tumor
type. Thus, only 38% of the BL tumors are EBV-positive compared with 65% of the
large-cell lymphomas.

**T cell lymphomas**

Recent studies have demonstrated EBV infection in a considerable proportion of T cell
non-Hodgkin’s lymphomas (Table 1). Nasal T cell lymphomas, a tumor which is more
common in the Far East, is invariably EBV-positive whereas around 20% of T cell
lymphomas arising at other sites (gastrointestinal, lung, lymph nodes) are associated with
EBV.

**Hodgkin’s disease**

Epidemiological studies originally suggested a possible role for EBV in the etiology of
Hodgkin’s disease (HD). Thus, elevated antibody titers to EBV antigens have been
detected in patients with HD and these increased antibody levels are present before the
diagnosis of disease. Furthermore, there is an increased risk of HD following IM. EBV
has been demonstrated in around 50% of HD cases with both viral nucleic acid
(DNA/RNA) and virus latent antigens localized to the malignant component of HD, the
so-called Reed–Sternberg cells and their variants. The association of HD with EBV is
age-related; pediatric and older adult cases are usually EBV-associated whereas HD in
young adults is less frequently virus-positive. The proportion of EBV-positive HD in
developing countries is high consistent with a greater incidence of HD in children and
more frequent prevalence of the mixed cellularity histiotype (Table 1). Although the
incidence of HD is relatively low (1–3/100,000 per year) this tumor is not geographically
restricted, making its association with EBV significant in world health terms.

**Transmission and Tissue Tropism**

The usual route of EBV transmission is via saliva, although rare cases of infections
transmitted by blood transfusion have been reported. EBV, measured by its ability to
transform B lymphocytes *in vitro*, can be detected in oropharyngeal secretions from IM
patients, from patients receiving immunosuppressive drugs and, at a lower level, from
normal asymptomatic EBV-positive individuals. These observations, together with the
fact that EBV-transformed LCLs *in vitro* tend to be poor producers of the virus and B
lymphocytes permissive of viral replication have not been demonstrated *in vivo*, suggest
that EBV replicates and is shed at epithelial sites in the oropharynx and/or from salivary
glands. This is supported by the demonstration of replicating EBV in the more
differentiated epithelial cell layers of oral ‘hairy’ leukoplakia, a benign lesion of the
tongue found in patients with AIDS. However, the inability to detect EBV in normal
epithelial cells and the demonstration that EBV can be completely eradicated by irradiation in bone marrow transplant recipients indicates that B cells are likely to be the main site of EBV persistence and may also represent the target of the virus in primary infection (Fig. 1).

The B lymphotropism of EBV is due to the expression of a specific cell surface receptor on B cells, the CD21 antigen or CR2, which can bind EBV and the C3d component of complement. The EBV-encoded membrane antigen (MA) is a 340 kDa glycoprotein which is expressed on the outer membrane of the virion and has been identified as the viral protein that binds to CR2. The expression of CR2 has also been demonstrated on immature thymocytes and follicular dendritic cells suggesting that these cell types may also be susceptible to EBV infection. The lack of CR2 expression on epithelial cells implies that an alternative receptor or mechanism is responsible for their infection. Under certain circumstances EBV can infect other tissues (i.e. smooth muscle, endothelial cells) but the relevance of this effect to primary and persistent virus infection is unknown.

Pathogenicity
As EBV strain variation does not appear to have a significant effect on the pathogenicity of the virus, attention has concentrated on the patterns of viral gene expression in the different virus-associated diseases. The *in vitro* ability of EBV to transform resting B cells into permanent LCLs is associated with the constitutive expression of a limited number of EBV-encoded latent proteins; the nuclear antigens EBNA 1, EBNA 2, EBNA3A/3B/3C and EBNA-LP and the latent membrane proteins, LMP1 and LMP2. Analysis of the expression and function of these viral gene products suggests that the EBNA 2 protein and LMP1 play central roles in EBV-induced cell transformation. EBNA 2 is a transcriptional regulator whereas LMP1 functions as a constitutively activated receptor.

The pattern of EBV latent gene expression varies among the different virus-related diseases. Thus, the proliferation of B cells characteristic of IM and XLP is associated with the expression of all the EBV latent proteins as seen in LCLs. However, in BL and NPC there is a downregulation of the EBV latent protein expression: only EBNA 1 is found in BL whereas in NPC both EBNA 1 and the LMPs are expressed. Recent studies suggest that the pattern of EBV latent gene expression in HD is similar to that in NPC. The large-cell lymphomas of post-transplant patients resemble LCLs in expressing the full range of EBV latent genes whereas the phenotypic heterogeneity of EBV-positive AIDS-related lymphomas is reflected at the EBV latent protein level providing a spectrum of tumors from LCL-like to BL-like. These different patterns of EBV latent gene expression are the result of different virus transcriptional programs influenced by factors such as methylation and the host cell environment. The downregulation of certain EBV latent proteins in BL, NPC and HD does not preclude a role for these gene products at an earlier stage in the oncogenic process. Thus, differences in viral protein expression appear to influence the pathogenicity of EBV as it relates to the development of the various virus-associated malignancies.

Clinical Features of Infection
Although the majority of primary EBV infections are asymptomatic, those resulting in IM can initiate a range of clinical symptoms which may last for weeks or even months. IM occurs predominantly in the adolescent and young adult with rare cases in infants and individuals of greater than 30 years of age. The acute illness is associated with a sore throat characterized by hyperplasia of lymphoid tissue in the oropharynx, fever and generalized lymphadenopathy. There is lymphocytosis accompanied by the presence of atypical lymphocytes. Enlargement of the spleen is found in around 50% of IM patients at 2–3 weeks after the onset of symptoms. Hepatosplenomegaly is occasionally found and is associated with elevated levels of serum liver enzymes. Resolution of these symptoms results in the establishment of the life-long EBV carrier state as seen after normal, asymptomatic primary infection. Failure of this to occur can precipitate a chronic active EBV infection where the symptoms of IM persist. The fatal IM associated with XLP takes a rapid course usually associated with progressive failure of the lungs, kidneys, liver and bone marrow due to an overwhelming infiltration of these organs with EBV-infected B cells.

In the endemic areas of Africa 60% of children with BL present with jaw tumors. These jaw tumors are common in younger BL patients whereas older children are more likely to present with abdominal tumors similar to those found in the sporadic form of the disease. Whereas bone marrow involvement is frequently found in patients with sporadic BL, it is rarely a feature of endemic BL. Over half of NPC cases present with a cervical mass resulting from lymphoid spread of the primary tumor from its common site of origin in the lateral nasopharynx (fossa of Rosenmuller). Common symptoms resulting from the location of the primary NPC tumor include nasal obstruction and bleeding as well as those due to malformation of the eustachian tube such as ear blockage, otitis media and conductive hearing loss. Lymphomas in patients with immunodeficiency can present at a number of different anatomic sites including the mediastinum, lungs, central nervous system and abdomen. The site of presentation and rate of development of these tumors appears to be influenced by the type of immunodeficiency. The lymphomas associated with patients on immunosuppressive therapy often develop within the grafted organ. Lymphadenopathy is the most common presentation of HD and in around 30% of patients is accompanied by fever, night sweats and weight loss.

Pathology and Histopathology
The polyclonal activation of B cells and resultant T cell response account for the pathology associated with IM which is evident in the lymph nodes, peripheral blood, liver and spleen. In IM and the lymphoproliferative disorders associated with immunosuppression EBV-infected B cells (immunoblasts) are found as infiltrates in solid organs as well as bone marrow. This is more pronounced in immunodeficient patients where the polymorphic B cell hyperplasia can evolve into a high-grade B cell lymphoma of immunoblastic or undifferentiated large cell type. The jaw tumors characteristic of endemic BL are usually associated with multifocal disease involving liver, kidney and gut. BL is classified as a high-grade malignant lymphoma of small noncleaved follicle center B cell type with a ‘starry sky’ morphology resulting from infiltrating histiocytes and macrophages. The EBV-associated anaplastic or undifferentiated type of NPC is an aggressive tumor which tends to metastasize to cervical lymph nodes. NPC has been
classed as a ‘lymphoepithelioma’ on account of the associated heavy T lymphoid infiltrate. The histopathology of HD is complicated by the paucity of the malignant Reed–Sternberg cells in tumor biopsies. Histological subtypes of HD reflect the cellular composition of the affected lymph nodes and may correlate with clinical features as well as with EBV association.

**Immune Response**

EBV illicits both humoral and cell-mediated immune responses in infected hosts. Primary infection with EBV is associated with the rapid appearance of antibodies to replicative viral antigens such as VCA, EA and MA with a later serological response to the EBNA proteins. In IM these responses are accentuated and are accompanied by autoantibodies such as rheumatoid factor as well as a heterophile antibody response directed against antigens on the surface of sheep erythrocytes. These autoantibodies are the result of EBV-induced polyclonal B cell activation. In the chronic, asymptomatic virus carrier, antibodies to VCA, MA and the EBNA are found, the titer of which remain remarkably stable. Of these antibodies those against MA are particularly important as they have virus neutralizing ability and can also mediate antibody-dependent cellular cytotoxicity. As discussed previously the levels of these EBV-specific antibodies are elevated in the different virally associated diseases.

As with other persistent viruses, cell-mediated immunity plays an important part in controlling EBV infection (Fig. 1). This is evidenced by the effect of immunosuppression on EBV-infected individuals which results in increased excretion of the virus and is associated with an increased risk of developing EBV-positive lymphomas. The development and maintenance of class I MHC restricted EBV-specific cytotoxic T lymphocytes (CTLs) is of particular importance in the control of virus infection (Fig. 1). Virus-specific CTLs are able to efficiently recognize the EBV latent antigens and thus prevent the unlimited proliferation of EBV-infected B cells. More recent work demonstrates that CTL responses to virus lytic antigens are a common feature of both primary and persistent EBV infection. Although impairment of CTL function is responsible for lymphomagenesis in immunosuppressed patients, the development of BL, NPC and HD is more complex. The growth and survival of these tumors in immunocompetent individuals implies that the tumor cells can evade EBV-specific CTL surveillance. This may be achieved by restricting EBV latent gene expression to those viral proteins not recognized by EBV-specific CTLs and/or by the downregulation of target cell antigens required for immune recognition such as class I MHC and lymphocyte adhesion molecules. Interestingly, EBNA1 which is consistently expressed in all EBV-associated tumors is not a target for CD8-positive CTLs.

**Prevention and Control of EBV Infection**

The importance of the EBV-associated malignancies in world health terms has prompted the development of a vaccine against viral infection. As the MA glycoprotein on the EBV virion is a target for neutralizing antibodies during normal infection, this molecule has been proposed as a suitable immogen for eliciting protective immunity. In the experimental cotton-top tamarin model, immunization with various preparations of MA is able to protect the animals against lymphomagenic doses of EBV. Recombinant soluble
MA has now been produced and clinical trials should take place in the next few years. An important patient group to target will be those children awaiting transplantation where virus-neutralizing antibodies raised in response to MA vaccination may provide protection from the development of EBV-associated lymphomas.

As the nature of primary EBV infection may make prophylactic vaccination extremely difficult, the use of therapeutic vaccines, perhaps directed to other EBV-encoded proteins, should be considered. The possibility of peptide vaccination with well-defined CTL epitopes is attractive particularly in NPC and HD where EBV latent gene expression is restricted. Adoptive CTL therapy using *ex vivo* expanded EBV-specific CTL preparations has proven successful in treating virus-associated lymphoproliferative disease and lymphomas in bone marrow transplant patients. A similar approach to the treatment of NPC and HD is currently being explored.

The antiviral agent acyclovir is a potent inhibitor of EBV DNA polymerase thus preventing virus replication. Acyclovir does not significantly alter the clinical course of IM or XLP. A few reports suggest that this drug used in conjunction with interferon may resolve the polyclonal B cell lymphomas found in transplant recipients but in this group withdrawal or reduction in immunosuppression is more effective. Although chemotherapy is extremely effective against BL, recent epidemiological evidence shows that the incidence of disease can be significantly reduced by malaria eradication. NPC can be successfully treated by surgery and local radiotherapy only at early stages of the disease. Early diagnosis using serologic screening for EBV-specific IgA antibodies and CAT scans has helped in identifying those patients with treatable disease. Combined chemotherapy is successful in 70% of HD patients. The EBV-positive form of HD may be more difficult to treat and thus the early identification of these cases using EBV-specific monoclonal antibodies could be useful.

**Future Perspectives**

The use of a variety of molecular virological techniques has unequivocally identified EBV as oncogenic in humans. However, the precise nature of primary infection and the subsequent establishment of life-long viral persistence is poorly understood and will require the further refinement of techniques for identifying individual EBV-infected cells *in vivo*. Any prophylactic vaccine must be effective at preventing primary infection and requires a better knowledge of the immune control of EBV infection and replication at the site of primary infection. More efficient *in vitro* systems for studying EBV infection and replication will help in this regard. The use of EBV recombinants has already yielded valuable information on the function of certain viral genes and will continue to help identify the cellular and viral factors responsible for tumor development. Understanding the host cell:virus interaction will be dependent on the generation of appropriate *in vitro* models which are currently lacking for NPC and HD. The application of adoptive CTL therapy for EBV-associated tumors will depend on a better understanding of the tumor microenvironment and may require approaches aimed at manipulating the local cytokine milieu. A more detailed knowledge of the functions of individual EBV latent proteins will allow the development of sophisticated pharmacological approaches to the treatment of virus-associated malignancies.
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IMMUNE RESPONSE: Cell Mediated Immune Response, General Features

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Figure Gallery

Figure 1 A model for EBV infection in normal healthy virus carriers. Primary EBV infection occurs in the oropharynx and is probably mediated via B cells. Following primary infection, a chronic virus carrier state is established in which EBV infection persists in resting B cells which may only express the EBNA1 protein. Occasional reactivation of EBV in this compartment results in the outgrowth of EBV-infected B cells which resemble EBV-transformed lymphoblastoid cell lines in vitro. The growth of these transformed cells is controlled by an EBV-specific cytotoxic T lymphocyte (CTL) response. At certain sites such as the oropharynx, latently infected B cells may become permissive for lytic EBV infection shedding infectious virus (i.e. into the saliva) thereby resulting in infection of other cells in the vicinity such as B cells and epithelial cells.
Marburg and Ebola viruses both cause severe hemorrhagic fevers. Marburg virus was first recognized in laboratory workers in Marburg, Germany, and Belgrade, Yugoslavia, in 1967. These workers had been exposed to tissues and blood from African green monkeys (Cercopithecus aethiops) imported from Uganda. There were 25 primary cases and six secondary cases in the outbreak. Seven of the primary cases died. Since then,
sporadic, virologically confirmed Marburg disease cases have occurred in Zimbabwe, South Africa and Kenya. Ebola virus first emerged in two major disease outbreaks which occurred almost simultaneously in Zaire and Sudan in 1976. Over 500 cases were reported, with mortality rates of 88% in Zaire and 53% in Sudan. A single case was confirmed by virus isolation in Zaire in 1977, and in 1979 Ebola hemorrhagic fever occurred again in Sudan at the site that was involved in 1976. In 1994 the first case of Ebola virus disease occurred in western Africa, Cote d’Ivoire, when an ecologist was infected by examining a dead chimpanzee. Ebola virus re-emerged in Kikwit, Zaire, in 1995, with 316 cases and 245 deaths. From 1994 to 1997 three outbreaks of Ebola virus disease have been observed in Gabon. Ebola-Reston virus was first isolated from naturally infected nonhuman primates in 1989–1990, when Cynomolgus monkeys were imported from the Philippines into the USA, and later from monkeys at an export facility located in the Philippines. Further isolates have been made from exported Asian monkeys in 1992 in Italy and in 1996 in Texas, USA. While pathogenic for naturally and experimentally infected monkeys, Ebola-Reston virus may be less pathogenic for humans, having infected four animal caretakers without producing serious disease. At least three laboratory infections with Marburg and Ebola viruses (two fatal outcomes) occurred in Russia; a single nonfatal laboratory infection (Ebloa) occurred in the UK.

**Taxonomy and Classification**

Filoviruses are classified in the order *Mononegavirales*, a large group of viruses that have nonsegmented negative-stranded RNA as their genomes. The family *Filoviridae*, genus *Filovirus*, was created on the basis of unique morphologic, physicochemical, genetic and biological features of its members. Filoviruses are separated into two distinct species, Marburg and Ebola. The Marburg species consists of a single subtype Marburg including five strains. The Ebola species is subdivided into four subtypes: Zaire, Sudan, Cote d’Ivoire and Reston. In terms of biohazard classification, filoviruses are classified as Biosafety Level 4 (BSL4) agents based on their high mortality rate, person-to-person transmission, potential aerosol infectivity, and absence of vaccines and chemotherapy. Maximum containment is required for all laboratory work with infectious material.

**Properties of the Virion**

By electron microscopy, filovirus particles are pleomorphic, appearing as long filamentous, sometimes branched forms, or as ‘U’-shaped, ‘6’-shaped or circular forms. The particles vary greatly in length (up to 14 000 nm), but have a uniform diameter of about 80 nm. Virions purified by ratezonal gradient centrifugation are bacilliform in outline and show an average length associated with peak infectivity of approximately 665 nm for Marburg and 805 nm for Ebola virus. Except for the difference in length, filoviruses seem to be very similar in morphology. Virions contain a nucleocapsid consisting of a dark, central space (20 nm in diameter) surrounded by a helical capsid (50 nm in diameter) bearing cross-striations with a periodicity of approximately 5 nm. Within the nucleocapsid is an axial channel of 10–15 nm (Fig. 1). The nucleocapsid is composed of the genomic RNA and the large (L) protein, nucleoprotein and virion proteins 35 and 30. It is surrounded by a lipoprotein unit membrane envelope derived from the host cell plasma membrane. Spikes of approximately 7 nm length, spaced at
approximately 10 nm intervals, are visible on the virion surface and are formed by the viral glycoprotein.

**Physical Properties**

Virus particles have a molecular weight of approximately 3–6 × 10^8 and a density in potassium tartrate of 1.14 g ml⁻¹. Uniform, bacilliform particles have a sedimentation coefficient of 1300–1400 S, whereas larger particles have a higher sedimentation coefficient. Virus infectivity is quite stable at room temperature. Inactivation can be performed by UV and gamma irradiation, 1% formalin, -propiolactone, and brief exposure to phenolic disinfectants and lipid solvents, like deoxycholate and ether.

**Properties of the Genome**

The genome of filoviruses consists of a molecule of linear, nonsegmented, negative-stranded RNA which is noninfectious, not polyadenylated, and complementary to viral-specific messenger RNA. The genome amounts to 1.1% of the total virion weight and the sedimentation coefficient is 46 S (0.15 mol l⁻¹ NaCl, pH 7.4). Filovirus genomes are approximately 19 kb in length and very rich in adenosine and uridine residues. Genomes show a linear gene arrangement in the order 3’ leader – nucleoprotein (NP) – viral structural protein (VP) 35 – VP40 – glycoprotein (GP) – VP30 – VP24 – polymerase (L) – 5’ trailer. All genes are flanked at their 3’ and 5’ ends by highly conserved transcriptional start (3’-CUnCnUnUAUU-5’) and termination (3’-UaAUUCUUUUU-5’) signal sequences, respectively, which almost all contain the pentamer 3’-UAAUU-5’.

Most genes are separated by intergenic sequences variable in length and nucleotide composition, but some genes overlap by the conserved pentanucleotide sequence. Subtype Zaire Ebola viruses show three overlaps that alternate with intergenic sequences (VP35/VP40, GP/VP30, VP24/L), while the Marburg virus genome contains a single overlap at a different position (VP30/VP24). Extragenic sequences are present at the 3’ and 5’ end of filovirus genomes which are complementary at their very extremities. These sequences are comparable to those found in genomes of other nonsegmented negative-stranded RNA viruses and are known as leader sequences. However, neither (+) nor (-) leader RNAs have been detected in filovirus-infected cells.

**Properties of Viral Proteins**

Virions contain at least seven proteins with presumed identical functions for the different viruses. The electrophoretic mobility patterns of the structural proteins are characteristic for Marburg strains on the one hand and Ebola strains on the other. Four proteins are associated with the viral ribonucleoprotein complex (NP, L, VP30 and VP35), the single glycoprotein (GP) is inserted in the envelope, and the location of two proteins (VP40 and VP24) has not been determined exactly, but they seem to be membrane associated. The L protein is the largest protein and, like other L proteins of nonsegmented negative-stranded RNA viruses, represents the virion-associated RNA-dependent RNA polymerase. Its size, as calculated from the deduced amino acid sequence of the Marburg virus (Musoke strain) L gene, is 267 kDa. GP (Marburg virus 170 kDa; Ebola virus 160 kDa) is a type I transmembrane protein and inserted in the lipid membrane as a homotrimer, as shown directly for Marburg virus. It is reasonable to assume that GP is the mediator of virus entry into the cell. Functional sites for receptor recognition and
binding, and perhaps for fusion, should be located on this protein. GP undergoes several post-translational modifications: glycosylation, acylation and proteolytic cleavage. The carbohydrate structures of this highly glycosylated protein account for >50% of its Mr. They include oligomannosidic and hybrid type N-glycans as well as bi-, tri- and tetra-antennary complex species, and high amounts of neutral mucin-type O-glycans. In contrast to Ebola viruses, sialylation of Marburg virus carbohydrate structures is cell line dependent. In case of Marburg virus GP, acylation occurs at cysteine residues located at the boundary between the membrane anchor and cytoplasmic domains. The Golgi-specific precursor (preGP) is proteolytically cleaved into the subunits GP1 and GP2 by a proprotein convertase, most likely furin. Mature GP (GP1/2) is a disulfide-linked complex of the cleavage subunits and is anchored in the membrane via the carboxy-terminal part of GP2. The major nucleoprotein (NP) (Marburg virus 96 kDa; Ebola virus 104 kDa) and VP30 (Marburg virus 28 kDa; Ebola virus 30 kDa), which may represent a minor nucleoprotein, seem to be intimately associated with the virion ribonucleoprotein complex. VP35 (Marburg virus 32 kDa; Ebola virus 35 kDa) is loosely associated with the ribonucleoprotein complex and seems to be a component of the transcriptase complex analogous to the P protein of paramyxoviruses and the NS (P) protein of rhabdoviruses.

The functions of VP40 (Marburg virus 38 kDa; Ebola virus 40 kDa) and VP24 (Marburg virus 24 kDa; Ebola virus 24 kDa) are not known, but they are probably membrane components. VP40 is the most prominent viral structural protein and most likely represents the matrix protein of filoviruses. A nonstructural glycoprotein has recently been discovered with Ebola viruses. This protein, designated sGP, is the primary gene product of gene 4 and efficiently secreted from infected cells. Recently it was detected in high concentrations in the blood of acutely infected Ebola hemorrhagic fever patients. The function of sGP is unknown, but an interaction with the cellular and humoral host immune responses has been postulated. A similar protein is not found with Marburg viruses.

Replication

Cell entry seems to be mediated by GP as the only surface protein of virion particles. Studies on Marburg virus infections of hepatocytes have identified the asialoglycoprotein receptor as a receptor candidate. However, one has to postulate additional receptors as this protein is not expressed on many virus-susceptible cells. Whether the next step in virus entry involves a fusion process at the plasma membrane or fusion following endocytosis of virus particles is not known. The genetics of filoviruses are probably similar to those of Paramyxoviridae and Rhabdoviridae. Transcription and replication take place in the cytoplasm of infected cells. The 3' leader of the genome probably provides the encapsidation site for the nucleoprotein as well as the entry site for the polymerase. Filovirus genomes are transcribed to yield mainly monocistronic subgenomic messenger RNA species which are complementary to viral genomic RNA. Transcription of the Ebola virus GP gene gives rise to at least two different messenger RNA species. The primary transcript encodes small (s) GP which is encoded by the amino-terminal open reading frame of gene 4. Full-length GP is expressed by transcriptional editing of a single nucleotide at a run of uridine residues that combines the sGP open reading frame with an overlapping additional reading frame in -1. The 5' ends of the subgenomic RNAs start precisely at the transcription start signal sequence, and the
3’ ends carry a poly (A) tail generated by the polymerase at a run of uridine residues located at the 5’ ends of all transcription termination signal sequences. Transcription efficiency might be influenced by gene order, formation of secondary structures at the 3’ ends of the genes, secondary structure formation within the intergenic sequences, overlapping genes, and presence of duplicated termination sites. Filovirus transcripts contain unusually long untranslated regions, especially at the 3’ ends. The 5’ end untranslated regions show a potential for a formation of stable hairpin structures, which might play a role in transcript stability and ribosome binding. Replication of the genome is mediated by the synthesis of a full-length complementary antigenome ( (+) sense) which then serves as the template for the synthesis of progeny negative-stranded RNA molecules anticomplementary to the parental template RNA. The complementarity of the genome extremities suggests a single identical encapsidation site on the genome and antigenome and an identical entry site for the polymerase for both the transcription and the replication mode. The cytoplasm of virus-infected cells contains prominent inclusion bodies consisting of viral nucleocapsid. As infection proceeds, they grow and become highly structured. Budding of completed virus particles takes place at cell membrane sites which are altered by insertion of the viral glycoprotein and alignment of viral membrane-associated proteins as well as of preformed nucleocapsids.

**Geographic Distribution**

Filoviruses, with the exception of subtype Reston of Ebola viruses, appear to be indigenous to the tropical rain forest regions of Central Africa, as indicated by the geographic locations of known outbreaks and seroepidemiological studies. This is also suggested by the fact that almost all filovirus isolates from human patients are of African origin. This includes the European isolates of Marburg viruses that could be traced back to foci in Uganda, from where vervet monkeys were imported to Germany and the former Yugoslavia. The Ebola-Reston outbreak suggested for the first time the presence of a filovirus in Asia. Studies among captive macaques in the Philippines indicated that the source of Ebola-Reston virus might be wild nonhuman primates; thus, it appears that filoviruses are not confined to Africa.

**Host Range and Virus Distribution**

The natural reservoirs for human and nonhuman primate infections with filoviruses are unknown. Experimental hosts include monkeys, for which infection with Marburg and Ebola-Zaïre virus are usually lethal, whereas some animals survive infections with Ebola-Sudan and Ebola-Reston viruses. Guinea pigs show febrile responses 4–10 days after inoculation with Marburg and Ebola viruses. However, none of these viruses kills guinea pigs consistently on primary inoculation. Ebola-Zaïre virus is usually pathogenic for newborn mice after intracerebral and intraperitoneal inoculation. For growth in cell culture, primary monkey kidney cells and monkey kidney cell lines, usually Vero cells, are used. Filoviruses also grow in human endothelia maintained as primary cell cultures or as organ cultures.

**Evolution**

Molecular analyses of the genomes clearly demonstrated that filoviruses are the closest relatives to *Rhabdoviridae* and *Paramyxoviridae*. All nonsegmented negative-stranded
RNA viruses share a similar genome organization, with conserved regions at both ends encoding the core and L proteins surrounding a variable part in the middle encoding the envelope proteins. Filovirus genomes are more complex than those of lyssaviruses and vesiculoviruses and align organizationally more closely to members of the genera Paramyxovirus and Morbillivirus. This relationship is confirmed on the amino acid level as demonstrated for the nucleoproteins and polymerases (L proteins). The Marburg species is genetically more homogenous. Comparative sequence analysis showed that two lineages coexist with the recent isolate from Kenya (Ravn strain, 1987) differing from the others by 21–23% at nucleotide level. Since this divergence is less than that separating Ebola subtypes (see below), the Ravn strain does not represent a distinct subtype. Significant differences within the Ebola species were first based on peptide and oligonucleotide mapping, which have since been confirmed by sequence comparison analysis of the glycoprotein genes. All four subtypes differ from one another by 37–41% and 34–43% at the nucleotide and amino acid level, respectively. Phylogenetic analysis of the glycoprotein open reading frame revealed a closer relationship of the three African subtypes compared with Reston, supporting the concept of an Asian origin of Reston viruses. Within individual subtypes of filoviruses the variation in nucleotide sequences has been shown to be <7% and even <2% among distinct subtype Zaire viruses. Recently it was reported that there is no genetic variability between strains isolated from different patients of single outbreaks in Gabon and Kikwit. All the data indicate a remarkable degree of stability over time for RNA viruses that are usually thought to be extremely variable. Furthermore, it appears that filoviruses might have evolved into specific niches and may reflect a similar divergence in the natural hosts, assuming they have coevolved.

Serologic Relationships
Comparison of the two species of filoviruses, Marburg and Ebola, showed similarities between amino acid sequences of the structural proteins. This finding indicates that the structural proteins have maintained similar structures and functions. Despite this amino acid similarity, there is no indication that there is any significant serological (antigenic) crossreactivity between Ebola and Marburg viruses, but the different subtypes of the Ebola species share common epitopes. Neutralization tests for Marburg and Ebola viruses have not yet been shown sufficiently reliable to enable determination of taxonomic relationships.

Epidemiology
The reservoir of filoviruses remains a mystery. Many species have been discussed as possible natural hosts. However, no nonhuman vertebrate hosts or arthropod vectors have yet been identified. Epidemiological data, obtained in association with the 1967 Marburg outbreak and the 1994 Côte d’Ivoire case as well as the Ebola-Reston episodes, suggested monkeys as a potential reservoir of filoviruses. However, the high pathogenicity of filoviruses, especially of Marburg virus and subtypes Sudan and Zaire of Ebola virus, for nonhuman primates does not support such a concept. Similarities in biological properties to other viral hemorrhagic fever agents, such as ‘Old World’ arenaviruses, favor a chronic infection of an animal that regulates survival of filoviruses in nature. This is in line with recent data on experimentally infected fruit and insectivorous bats that demonstrated asymptomatic Ebola virus replication in these animals. Nosocomial
transmission, mainly due to a lack of hygiene, is of major public health concern. Based on experiences of former episodes, isolation of patients and use of strict barrier nursing procedures (e.g. protective clothing, respirator) are sufficient to interrupt transmission.

**Transmission and Tissue Tropism**
The mode of primary infection with Marburg and Ebola viruses in any natural setting is not known. The physical contact route of infection is undoubtedly the most common means of transmission from humans to humans. Especially activities such as nursing and preparing bodies for burials are associated with an increased risk of becoming infected. One Marburg case was acquired by sexual contact more than 60 days after the original infection. Neonatal transmission has been reported for the 1976 outbreak in Zaire. Transmission by droplets and small-particle aerosols has been observed among experimentally infected and quarantined imported monkeys (Ebola-Reston virus, 1989–1990). This is confirmed by identification of filovirus particles in alveoli of naturally and experimentally infected monkeys. However, the contribution of aerosol transmission to the course of human outbreaks is still unknown.

Virus is usually recovered from acute-phase sera and has also been found in throat washes, urine, soft tissue effusates, semen and anterior eye fluid, even when the specimens were obtained late in convalescence. It has also been regularly isolated from autopsic material, such as spleen, lymph nodes, liver and kidney, but rarely from brain or other nervous tissues.

**Pathogenicity**
Marburg and Ebola viruses cause severe hemorrhagic fever in humans and laboratory primates. According to the evidence available to date, subtype Reston of Ebola virus causes hemorrhagic fever in monkeys, but appears to be apathogenic for humans. The subtype Zaire strains of Ebola virus apparently have the highest mortality in humans when compared with other subtypes or Marburg virus.

**Clinical Features of Infection**
Clinical symptoms are similar with Marburg and Ebola virus infections. Following incubation periods of 4–16 days, onset is sudden, marked by fever, chills, headache, anorexia and myalgia. These signs are soon followed by nausea, vomiting, sore throat, abdominal pain and diarrhea. When first examined, patients are usually overtly ill, dehydrated, apathetic and disoriented. Pharyngeal and conjunctival injections are usual. Most of the patients develop severe hemorrhagic manifestations, usually between days 5 and 7. Bleeding is often from multiple sites, with the gastrointestinal tract, lungs and gingiva the most commonly involved. Bleeding and oropharyngeal lesions usually herald a fatal outcome. Death occurs between days 7 and 16, usually from shock with or without severe blood loss.

**Pathology, Histopathology and Pathogenesis**
Marburg and Ebola viruses cause similar pathological changes in humans. The most striking lesions are found in liver, spleen and kidney. These lesions are characterized by focal hepatic necrosis with little inflammatory response and by follicular necrosis of
lymph nodes and spleen. In late stages of the disease, hemorrhage occurs in the gastrointestinal tract, pleural, pericardial and peritoneal spaces, and into the renal tubules with deposition of fibrin. Abnormalities in coagulation parameters include fibrin split products and prolonged prothrombin and partial thromboplastin times, suggesting that disseminated intravascular coagulation is a terminal event. There is usually also profound leukopenia in association with secondary bacteremia. Ebola-Reston virus causes similar pathological changes in monkeys, as described for human infections with Marburg and other subtype Ebola viruses. In Reston-infected animals it was clearly demonstrated that virus replication was extensive in fixed tissue macrophages, interstitial fibroblasts of many organs, circulating macrophages and monocytes, and less frequently in vascular endothelial cells, hepatocytes, adrenal cortical cells and renal tubular epithelium. Macrophages seem to be the first and preferred site of replication by filoviruses.

Clinical and biochemical findings support anatomical observations of extensive liver involvement, renal damage, changes in vascular permeability, and activation of the clotting cascade. Visceral organ necrosis is the consequence of virus replication in parenchymal cells. However, no single organ is sufficiently damaged to explain fatal outcome. Fluid distribution problems and platelet abnormalities indicate dysfunction of endothelial cells and platelets. Virus-induced release of humoral factors, such as cytokines, may increase endothelial permeability and may be a major factor in the shock syndrome regularly observed in severe and fatal cases.

**Immune Response**

Fatal filovirus infections usually end with high viremia and no evidence of an effective immune response. In monkeys infected with Ebola-Reston virus nonprotective antibodies have been observed shortly before death. Altogether, however, the data available today do not support an important role of neutralizing antibodies in virus clearance. Since circulating monocytes/macrophages are primary target cells in filovirus infections, and since the extensive disruption of the parafollicular regions in spleen and lymph nodes results in the destruction of the antigen-presenting dendritic cells, cellular immunity appears also to be affected during filoviral hemorrhagic fever. In addition to these cytolytic effects, there are several other mechanisms by which filoviruses may interfere with the immune system. Firstly, the high carbohydrate content of GP may suppress its immune reactivity. Secondly, the nonstructural sGP that is secreted from cells infected with Ebola virus may interfere with the virus-directed immune response. Thirdly, GP1 which is also secreted in large amounts from infected cells, may have similar effects. Fourthly, full-length GP displays a sequence motif homologous to an immunosuppressive domain observed in retroviral glycoproteins. Altogether, there is now a large body of evidence indicating that filoviruses induce immunosuppression in the infected host, which appears to be a major factor for the rapid spread and the severity of the disease.

**Prevention and Control**

Although neutralizing antibody titers in human convalescent sera can, if at all, only barely be detected in laboratory tests, there are anecdotal case reports suggesting the potential benefit of passive immunization against Ebola virus infection. Furthermore, recent reports from the 1995 outbreak in Zaire about effective treatment of acutely ill
patients with whole blood transfusions from convalescent donors suggest that quantities of antibodies, predicted to be marginally effective in laboratory tests, may still be protective. There is experimental evidence that active immunization employing killed virus, recombinant expressed glycoprotein, and recombinant gene 4 (GP)-DNA (DNA vaccination) is partially successful in animals, suggesting that these may be feasible strategies to elicit protective immunity. At present, however, vaccines for human application are not available. A specific chemotherapeutic treatment is not available to date, but knowledge of the expected clinical course can anticipate medical complications, including disseminated intravascular coagulation, shock, encephalomyelitis, cerebral edema, kidney failure, superinfection, hypoxia and hypotension. Patients have to be isolated and clinical personal to be protected. Human interferon, human convalescent plasma and anticoagulation therapy has been used, but their success is still controversial.

**Diagnosis**

Because Marburg and Ebola viruses are highly virulent, special precautions need to be taken when samples are handled, and for some procedures biocontainment (BSL4) is necessary. For acute diagnosis polymerase chain reaction (PCR) and antigen-ELISA are suitable. For confirmation virus isolation should be initiated in appropriate cell cultures (Vero E6 cells, MA 104 cells) from acute-phase serum or biopsy/autopsy materials (liver, spleen, lymph nodes, kidney, heart). During viremia particles can usually be visualized by electron microscopy. Serum antibody titers are determined by ELISA (IgM -capture, IgG) or indirect fluorescent-antibody immunofluorescence assay (IFA) (caution: prone to nonspecific positives). Serum from patients with suspected cases should be inactivated by gamma irradiation prior to serotesting. Neutralization tests are totally unreliable for filoviruses.

**Related Topics**

**Emerging and Re-emerging Virus Diseases**

**Further Reading**


**Figure 1** Marburg virus particles. An electron micrograph (ultrathin section) showing budding of Marburg virus particles from the plasma membrane of infected primary cultures of human endothelial cells. Particles consist of a nucleocapsid surrounded by a membrane in which spikes are inserted (arrows). The nucleocapsid contains a central channel (inset). The plasma membrane of infected cells is often thickened at locations where budding occurs (arrowheads). Bar = 0.5 m; inset bar = 50 nm.